

Abstract

This project was carried out in the field of biomaterials, in the section regenerative therapies and precisely aimed at tissue engineering.

Bone restoration and regeneration are a major importance in the tissue engineering field. Basically, an implant such as scaffolds for bone regeneration has to stimulate osteogenesis and angiogenesis, apart from being bioresorbable as well. Nevertheless, biomaterials for osteogenesis and angiogenesis should be considered not only as a template for cell growth, but also as a delivery agent. Indeed, previous and current studies aimed in this direction, promotes the key role of calcium in blood vessel formation.

The interesting achievement is to obtain nanostructured materials by electrospinning with embedded calcium and phosphate compounds, which shall deliver at an appropriate rate. In our case, the study was directed to bioactive glass nanoparticles, whose purpose is to be incorporated in polymeric fibers. In order to reach desired compositions, the particles were synthesized by the sol-gel process. A first glass synthesis was performed to acquire the "G5", whose composition is known to be as: 44.5% P_2O_5 , 44.5% CaO, Na₂O 6% 5% TiO_2 . However, the main task of this project was to produce new bioactive glass nanoparticles using different precursors: especially calcium propionate and phytic acid to obtain ternary glass particles with a desired composition of 47.5% P_2O_5 , 47.5% CaO, 5% TiO_2 . Indeed, the challenging nature of the study was to use precursors whose byproducts appear to be biocompatible and biodegradable. The current precursors used, for the fabrication of the regular "G5" particles, contain whether ethanol or 2-methoxyethanol, known to be cytotoxic once released in the body. New precursors whose byproducts after release can be eliminated by the body would an important progress.

The difficulty in the control of sol-gel parameters according to the difference of precursors have shown quite broad range of composition which have been compared to the defined G5 glass. Further studies have to be completed in order to define an effective protocol capable of producing these nanoparticles with better control in terms of compositions and morphology, because it is precisely the first features which affect the additional properties of our material, such as pH, calcium release and of course cell proliferation.

Table of contents

ABSTRACT	1
TABLE OF CONTENTS	3
1. GLOSSARY	5
2. PREFACE	7
3. INTRODUCTION	9
4. BACKGROUND THEORETICAL	10
4.1. Regenerative medicine and tissue engineering	10
4.2. Biomaterials	11
4.3. Biomaterials for osteogenesis and angiogenesis	14
4.3.1. Nanostructured materials produced by electrospinning	15
4.3.2. Calcium-phosphate based glasses	17
4.3.3. Sol-gel process	19
4.3.4. Controlled release glasses (CRG)	26
4.3.5. Impact on biocompatibility – Cytotoxicity of byproducts	27
4.4. Objectives	30
5. MATERIALS AND METHODS	31
5.1. Synthesis of the glass nanoparticles	31
5.2. Characterization methods	38
5.2.1. Scanning Electron Microscopy (SEM)	38
5.2.2. Degradation	39
5.2.3. X-Ray Diffraction	43
5.2.4. Zeta potential and size measurements of particles	43
5.3. Cell culture	45
5.3.1. Cell seeding	46
5.3.2. AlamarBlue® - Proliferation tests	47
6. RESULTS AND INTERPRETATIONS	50
6.1. Chemical Composition	50
6.2. Morphology of the particles (Shape and Size)	55
6.3. Zeta potential analysis	60
6.4. Crystallinity of the particles	62

6.5. Degradability	65
6.6. Cell assays – Proliferation tests	68
7. CONCLUSION	71
ACKNOWLEDGEMENT	73
SPENDING	75
ENVIRONMENTAL EVALUATION	78
BIBLIOGRAPHY	79
Bibliographical references	79

1. Glossary

G5 Glass composition are identified as GX (G standing for Glass and X corresponding to the atomic percentage of titanium in the glass composition)

Sol-gel process Chemical synthesis technique for preparing gels, glasses, and ceramic powders.

P₂O₅ Phosphorus pentoxide

CaO Calcium oxide

TiO₂ Titanium dioxide

Na₂O Sodium oxide

Biocompatibility Ability of a material to fulfill its function resulting in an appropriate response of the receiver organism in a specific situation

Bioresorbable Gradual degradation in the body over a period of time (do not require mechanical removal)

Bioactivity Ability to interact directly with a living tissue

Osteoproduktive Ability of allowing the colonization of a bioactive surface with osteoblasts – it causes both intra- and extracellular response on its interface

Osteoconductive Just a biocompatible surface above which bone cells can migrate – it causes just an extracellular response

Scaffold Provide the structural support for cell attachment and subsequent tissue development

Angiogenesis The process of developing new blood vessels

Osteogenesis The process of laying down new bone material by osteoblasts

Electrospinning Technique that use an electric charge to pull very fine fibers from a liquid

PLA Poly (lactic acid) polymer – molecular formula $(C_3H_4O_2)_n$

CaP Calcium phosphate

BG Bioglass® - a commercially available family of bioactive glasses

HA Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$

Alkoxides A compound formed from an alcohol by the replacement of the hydrogen of the hydroxyl group with a metal

Hydrolysis A chemical reaction in which the interaction of a compound with water results in the decomposition of that compound.

Condensation A chemical reaction in which two molecules react with the resulting loss of a molecule of water (or other small molecule); the formal reverse of hydrolysis.

Hybrid Combination of organic materials and inorganic materials

Byproducts Compounds produced during the degradation of the material

SEM Scanning Electron Microscopy

EDX Energy Dispersive X-ray (Spectroscopy)

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid – chemical buffering agent

DRX X-ray Diffraction. Technique for characterization of crystalline phases

rMSCs Rat mesenchymal cells

AlamarBlue® A method to evaluate the evolution of the cell population

TFE Trifluoroethanol

D.b.d (Recap chart) Drop by drop

2. Preface

The present study was carried out as a Master Thesis internship and includes the six-month internship in laboratory required in order to achieve the first semester of the 5th and final academic year in engineering and materials science at the EEIGM Nancy, European School of Engineering and Materials Science.

The internship was performed at the Institute for Bioengineering of Catalonia, located in the Scientific Park of Barcelona – Spain, from September 2013 to February 2014. The Institute for Bioengineering of Catalonia (IBEC) is an interdisciplinary research center focused on bioengineering and nanomedicine, based in Barcelona. It has several areas of research such as:

- Cellular Biotechnology
- Biomechanics and cellular biophysics
- Nanobiotechnology
- Biomaterials, implants and tissue engineering
- Medical signals and instrumentation
- Robotics and biomedical imaging

I was involved during this project in the Biomaterials, implants and tissue engineering research program. It concerns the development of new materials and structures for biocompatible replacement, tissue formation and technologies for repairing or replacing tissues and organs. My work took place in the group directed by Dr. Elisabeth Engel, focusing on biomaterials for regenerative therapies, under the supervision of Dr. Oscar Castaño.

3. Introduction

Tissue engineering and regenerative medicine is an emerging multidisciplinary field involving biology, medicine, and engineering. It is aimed to restore structure and function of damaged tissues and organs by initiating the natural regeneration process. It applies both engineering and biology field in order to develop biological substitutes, which restore, maintain or improve tissue function.

As third-generation biomaterials, the use of scaffolds for bone regeneration intent to replace the damaged tissue and activate its regeneration, while being degraded to make room for new tissue. In this way, previous studies suggest that biomaterials for osteogenesis and angiogenesis should be considered also as ion delivery agent, as well as a template for cell growth. Therefore, as we know that calcium plays a key role during the blood vessels formation process, we will try to produce nanostructured materials using the electrospinning technique, in which calcium and phosphate must be integrated and most importantly shall deliver ions at a suitable rate.

This practical internship has been realized at the Institute for Bioengineering of Catalonia in the group directed by Dr. Elisabeth Engel under the supervision of Dr. Oscar Castaño. It was oriented towards the production and characterization of nanoparticles for hybrid nanofibers obtained via the sol-gel process using new precursors. This work tries to prevent cytotoxicity of the byproduct organic part of the material once implanted in the body with the use of two major new precursor which are calcium propionate and phytic acid. First of all, attempts will be performed to find suitable methods and protocols in order to obtain convenient compositions and morphology. Characterization standard methods on these $\text{CaO-P}_2\text{O}_5\text{-TiO}_2$ particles will be carry out so that we can foresee the impacts on the cytotoxicity of our byproducts, as well as an appropriate ions delivery rate and a possible incorporation into the polymeric matrix. This project appears to be quite challenging according to the fact that no previous work relate the desired incorporation of organic/inorganic hybrid nanoparticles into the human body.

4. Background theoretical

4.1. Regenerative medicine and tissue engineering

The promising field of regenerative medicine has several purposes. The main one is working to restore structure and function of damaged tissues and organs. To create solutions for organs that become permanently damaged is also one of the goals that regenerative medicine tries to reach; it is in other words “finding a way to cure previously untreatable injuries and diseases” [1].

The current research studies are working to make those treatments available for clinical use. There are actually 3 different main paths in the field of regenerative medicine:

- Artificial organs and medical devices
- Tissue engineering and biomaterials
- Cellular therapies

In our case, the main focus concerns tissue engineering and biomaterials. The goal of this part of regenerative medicine is to be able to maintain the body in such a way there will be no need to replace whole organs. Tissue engineering is a field in which it is possible to cover various dimensions of special applications designed to the recovery or the replacement of part of whole tissue as blood vessels, bones, cartilages, ligaments, etc... [2]

This field of tissue engineering applies the principles of engineering and life sciences toward the development of biological substitutes that restore maintain or improve tissue function [3]. It is difficult to be more precise when defining the field of tissue engineering, but according to Charles A. Vacanti, it can be defined as “the use of a natural or synthetic biodegradable materials, seeded with living cells when necessary, for regenerating the shape and/ or function of a tissue or of a damaged or diseased organ in a patient human” [4].

4.2. Biomaterials

One of the most accepted definition for biomaterials is one that has been employed by the American National Institute of Health: “any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual” [5].

Biomaterials are designed to interact with biological systems. To be able to be used and implanted “in vivo”, as to guarantee a good body tolerance and good results, those biomaterials must meet several requirements. Keys factors in a biomaterial usage are its biocompatibility and biofunctionality.

- Biocompatibility: the reaction between the implant and the body has to be studied, regarding both local and general reactions (the cytotoxicity of the material). But the influence of the body on the material has to be also considered. These reactions can eventually lead to degradation and loss of function [6]. In any case, we can foresee an initial inflammatory response, which can be essential in the healing process. However, prolonged inflammation can indicate an incompatibility.
- Biofunctionality: creating positive interactions to ensure continuity of the material and its environment, as well as providing additional performance to the product interface.

According to G Heness and B.Ben-Nissan [7], when you place a synthetic material within the human body, the tissue reacts towards the implant material in several different ways depending precisely on the material type itself. The mechanism of tissue interaction depends on the tissue response to the implant material surface. Three main denominations in which a biomaterial may be classified as to embody the tissue responses: Bioinert, Bioresorbable and Bioactive.

- Bioinert: this refers to a kind of material that once placed in the body has minimal interaction with the tissue. Alumina, stainless steel, stabilized zirconia are examples of bioinert materials [8].
- Bioresorbable: bioresorbable materials are designed to degrade gradually over a period of time and to be replaced by the natural host tissue. This leads to a very thin or nonexistent interface thickness. If the requirements of strength and short term performance can be gathered, bioresorbable implant are the optimal materials, as natural tissue can repair and/or replace themselves during life. Common examples are tricalcium phosphate [9].
- Bioactive: the concept of bioactivity is defined as “a bioactive material is one that elicits a specific biological response at the interface of the material which results in the formation of a bond between the tissues and the material” [10]. It is an intermediate between bioresorbable and bioinert. There are two classes of bioactive materials (Figure 1): Class A osteopductive materials (class A bioactivity occurs when a material elicits both an intracellular and an extracellular response at its interface – class A bioactive glasses can bond with both bone and soft tissue). Class B osteoconductive materials. The osteoconductive implant simply provides a biocompatible interface along which bone migrates (osteoconductive bioactivity occurs when a material elicits only an extracellular response at its interface [11].)

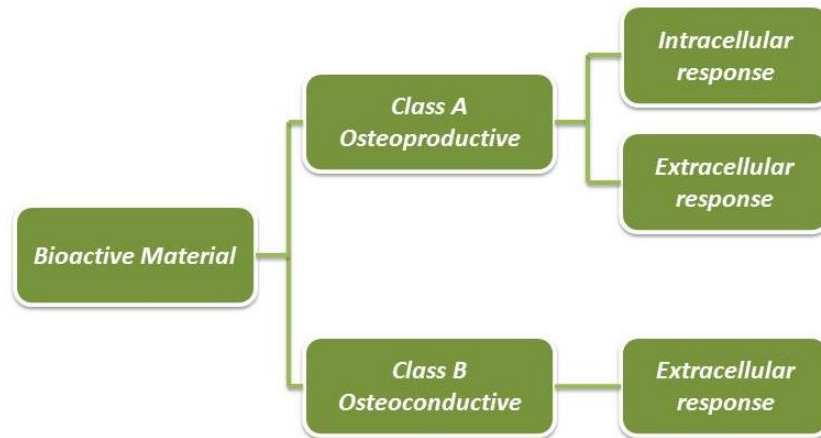


Figure 1 - Class A and B - Bioactive materials

Current studies intent to enhance production of materials that not only are bioactive but bioresorbable as well. This kind of materials needs to preserve the function of the tissue substituted during a desired period of time. If that function is regeneration, it has to stimulate a specific cellular response. As mentioned before, it must gradually degrade itself until it disappears completely from the body in order to make place for the new tissue.

A few examples of tissue attachment of biomaterials are given in the following table (Table 1) [12]:

Type of implants	Type of attachments	Example
Nearly inert	Mechanical interlock	Metals, Alumina, Zirconia, PE
Porous	Ingrowth of tissues into pores	Hydroxyapatite (HA)
Bioactive	Interfacial bonding with tissues	Bioactive glasses, HA, glass ceramics
Resorbable	Replacement with tissues	Tricalcium phosphate, PLA

Table 1 - Types of tissue attachments of biomaterials [12]

4.3. Biomaterials for osteogenesis and angiogenesis

The engineering of bone tissue offers various strategies to help musculoskeletal healing. New scaffolds implants have been designed in order to develop tissue engineered bone (Figure 2 [13]). However an active blood vessel network is a key for those scaffolds to integrate with existing host tissue [14]. The connective therapies of stem cells and polymeric growth factor are made for scaffolds to promote angiogenesis and osteogenesis; this is currently being developed to actively stimulate bone regeneration.

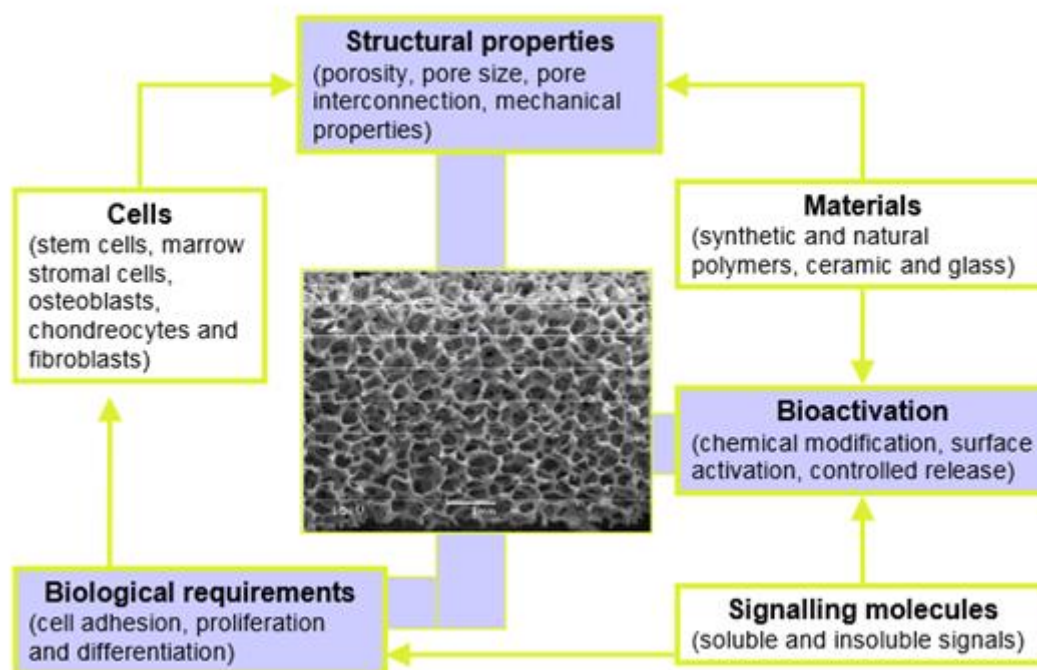


Figure 2 - Most important factors involved in the design of optimal scaffolds for bone tissue engineering [13]

As J.M. Kanczler and R.O.C. Oreffo described it, the role of angiogenic and osteogenic factors in the adaptive response and interaction of osteoblasts and endothelial cells during the multi steps process of bone development and repair [...], with consideration of how some of these key mechanisms can be combined with new developments in tissue engineering to enable repair and growth of skeletal fractures [11].

For the implant to play its role in the restoration of bone, it is essential that it integrates and interacts in a good way with its environment. In order to do so, an effective

cell adhesion and control on the surface are required. This process must be performed first with the proteins in the medium; they are the main compounds to reach the implant indeed. Protein response therefore depends on its surface energy, the chemical response of the surface, as well as topography.

One of the other important points in the design of a scaffold for bone regeneration is the need to allow adequate vascularization in the implant [28]. Angiogenesis is actually an essential process in the colonization of the biomaterial during the osteointegration operation. In this way, capillary transport the nutriments and oxygen necessary to allow their growth. In the case of this project, previous studies have shown that bioactive glasses can play an important part in the angiogenesis process; they can stimulate the secretion of angiogenic growth elements, but also endothelial cell proliferation.

4.3.1. Nanostructured materials produced by electrospinning

Electrospinning is a versatile technique which recently has significant development in nanofibers production. In this technique, a high electric field is applied to the droplet of a fluid, which in most of the studied cases is a solution (it comes out from the tip of a syringe, acting as one of the electrode). By doing so, we basically deform the droplet and this leads to the ejection of a charged jet from the tip of the cone accelerating towards the counter electrode and then leading to the formation of continuous fibers [15] (Figure 3).

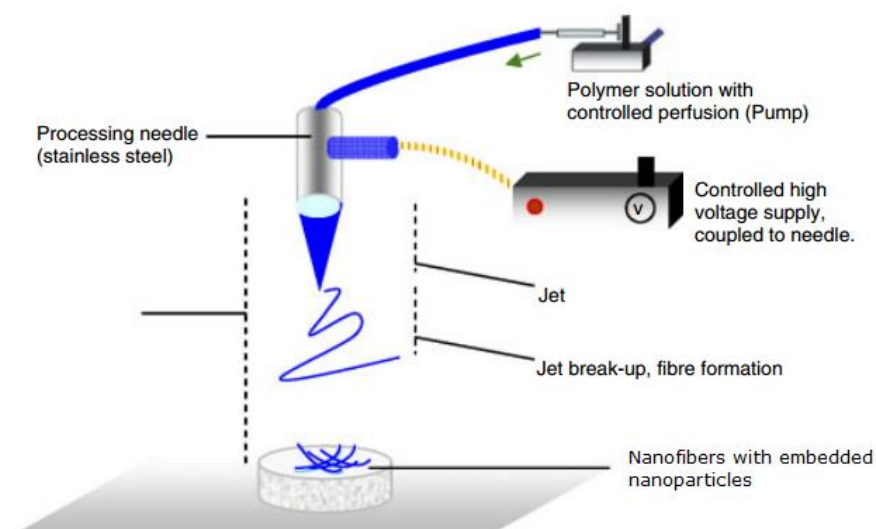


Figure 3 - Electrospinning schematic set-up [15]

The PLA polymer (polylactic acid) for example, has appeared to be a suitable material for tissue engineering and met almost all the requirements to be used as a scaffold; nontoxic behavior, biodegradable and bioresorbable but so far no bioactivity [28]. In order to get this bioactive behavior, it has to be combined with bioactive molecules or materials such as Bioglass® or more generally phosphate calcium based glasses (osteoconductive and able to bond to bone).

When you actually add those CaPs or BG particles, our scaffold is subjected to a degradation process problem, especially about the reduction of acidic degradation. In order to optimize the final scaffold, one technique has shown very good results: electrospinning.

Different properties of nanofibers can be obtained or adjusted by controlling the different parameters of the electrospinning process in order to obtain electrospun scaffolds for biomedical applications. A few applications are described: wound dressing, drug delivery, blood vessels, bone tissue engineering, etc... [15]

Wound healing: nanofibrous dressings using the electrospinning process have several advantages compared to others processes. Higher surface areas, higher microporous structure, better attraction of fibroblasts to the derma layer (that can release important extracellular matrix components) are properties encountered in wound healing electrospun materials (collagen, polyvinylalcohol PVA, gelatin, chitosan, and more nanofibrous materials).

Drug delivery: electrospinning provides a good feature when it comes to choose materials for drug delivery. The desired fibers can be oriented or arranged according to our main needs, they can control the mechanical properties and the biological response of the scaffolds (fibers oriented randomly). One of the very important positive points is that there are several different drug loading methods (coatings, encapsulated drug, and/or embedded drug for example). The attention given especially to those drug delivery systems is based on one essential advantage: reduced toxicity by delivering drugs at a controlled rate.

Blood vessels: the basic materials of the extracellular matrix locally around the vascular cells are a combination of type I and type III collagen, as well as elastin, and some proteins. Previous studies have shown artificial artery based on collagen scaffolds; nanomaterials development contributes to improve tissue engineering because of its particular nanostructure similarity to native extracellular matrices.

Bone tissue engineering: natural bone is a biocomposite made of both inorganic (HA crystals) and organic (collagen matrix) materials. In order to follow the matrix role, electrospinning technique appears to support quite easily the production of very fine and continuous nano/micro fibers. PLA matrix and CaPs/BG glasses are in a good example of designed scaffolds in bone tissue engineering, obtained by different ways (sol-gel method for the nanoparticles and electrospinning process for the nanofibers).

4.3.2. Calcium-phosphate based glasses

A current direction in the biomaterials field is to move from inert, passive materials to those that degrade and play an important active role in the regeneration process of the tissue. Implant materials are currently being developed in order to support and stimulate a specific biological response inside the body. As described in one paper [16], this new division of materials is often referred as the “3rd generation of biomaterials”, and with no surprise includes largely the phosphate-based glasses.

Bioactive glasses, and especially the ones containing silica, have been studied quite largely in various studies as materials for tissue regeneration application. Indeed, these studies have expressed some good opinions for “in vivo” cases. Basically when those bioactive glasses are exposed to physiological fluids, they tend to form a surface apatite layer, which is capable of bonding to collagen synthesized by connective tissue cells (osteoblasts) [17]. One type of bioactive glasses is currently available commercially as Bioglass® (45S5 – composition of 45 wt% SiO₂, 24.5 wt% CaO, 24.5 wt% Na₂O and 6.0 wt% P₂O₅). However, silica based bioactive glasses are recently discussed, especially concerning their slow degradation (often taking 1 to 2 years to disappear from the body [18]). Therefore, studies have been undertaken to search for bone repair and have led to phosphate based glasses as interesting alternatives.

Biodegradable scaffolds are beneficial products for tissue engineering applications. As an example we can name the CaO-Na₂O-P₂O₅ glasses, which show good properties in the use for hard tissue substitutes or synthetic graft materials. Phosphate glasses can also be doped with a few different metal alkoxides in order to change and modify their physical properties [19].

In our case, the synthesis for producing such bioactive phosphate based glasses is performed by using the sol-gel method, which has shown very good results in producing bioactive nanoparticles glasses for the biomedical applications.

4.3.3. Sol-gel process

Introduction to sol-gel method:

The sol-gel is a low temperature wet-chemical technique for the fabrication of oxide materials. The whole process begins with a chemical solution in order to produce colloidal particles; this is what we call the sol. The usual precursors are inorganic alkoxides and metal chlorides. They undergo hydrolysis and condensation reactions to create the colloid, this sol then evolves (after condensations reactions) towards the formation of an inorganic network containing a liquid phase; the gel. The growth of an inorganic network is due to the formation of M-O-M and M-OH-M bonds (where M is an electropositive element such as Si, Ti, and Al...). A drying step helps to remove the liquid phase and is then able to produce a porous material. A thermal treatment can be applied to enhance polycondensations, which means consolidation and densification of our material structures. One of the very important advantages of the sol-gel method is versatility: the precursor sol can be for example deposited on a substrate, cast with a desired shape, or used to synthesize ultra-fine powders [20].

Advanced theory of the sol-gel process:

The first sol-gel polymerization was described by Ebelmen, who introduced it in 1846 as “under the influence of atmospheric humidity a silicon alkoxide changed from a clear liquid into a transparent solid which on heating formed silicon dioxide.” However, the beginning of the sol-gel polymerization started in the 1930's for the use of the German company Schott, who used it for making glass containers. The sol-gel method then became an important research area for ceramic materials, not only for industrial purposes, because it offers the possibility to tailor your material according to purity, homogeneity, low temperature preparations, and direct molding [21].

. The oxide network formation takes place in solution, usually at a temperature close to room temperature. This is a conversion process in solution of metal alkoxides, such as silicon, zirconium, aluminum, titanium alkoxides... Although it is possible to use other derivative, for example chlorides, alkoxides are by far the most widely used because of their moderate reactivity and modularity. Indeed, the choice of the alkyl group enables modulation of the alkoxide reactivity based on the considered final properties for the material [21].

The ability to obtain different rheology (sols, gels or precipitates) is determined by the kinetics of hydrolysis and condensation reactions (Table 2), which is one of the major interests of the sol-gel:

Hydrolysis	Condensation	Rheology
Slow	Slow	Sol
Fast	Fast	Gel precipitate
Fast	Slow	Gel
Slow	Fast	Precipitate

Table 2 - Different types of products obtained by sol-gel process in terms of hydrolysis and condensation kinetics steps [23]

Sol-gel polymerization aspect

The sol-gel polymerization can be “hydrolytic”, meaning that it requires the addition of water and therefore include one or more hydrolysis steps, or can be “non-hydrolytic” when made without water. The hydrolytic gels case is by far the largest and most widespread.

In this case, the sol-gel includes at least one hydrolysis step prior to polymerization. We can describe the reaction in two steps: hydrolysis of the alkoxide, followed by condensation.

Hydrolysis

During this first reaction, hydroxyl functions are formed around the metal cation, making the precursor even more reactive to the condensation reaction. It is defined by the following chemical equation: $M(OR)_n + x H_2O \rightarrow M(OR)_{n-x}(OH)_x + x ROH$

The hydrolysis (Figure 4) starts by a nucleophilic substitution on the metal atom – step 1 with a proton transfer – step 2. An alkyl group is then removed as an alcohol – step 3.

The hydrolysis is more favored as:

- The incoming molecule is nucleophilic : $\delta(H_2O) > 0$
- The metal center is electrophilic : $\delta(O) \ll 0$; $\delta(M) \gg 0$
- The leaving group is nucleofuge : $\delta(ROH) \gg 0$

It should be noted that alkoxides and water are not miscible. Consequently these reactions take place in a common solvent, which generally corresponds to the alcohol generated during the hydrolysis but not necessarily. Indeed, several polar and water-miscible solvents have been used for sol-gel polymerizations.

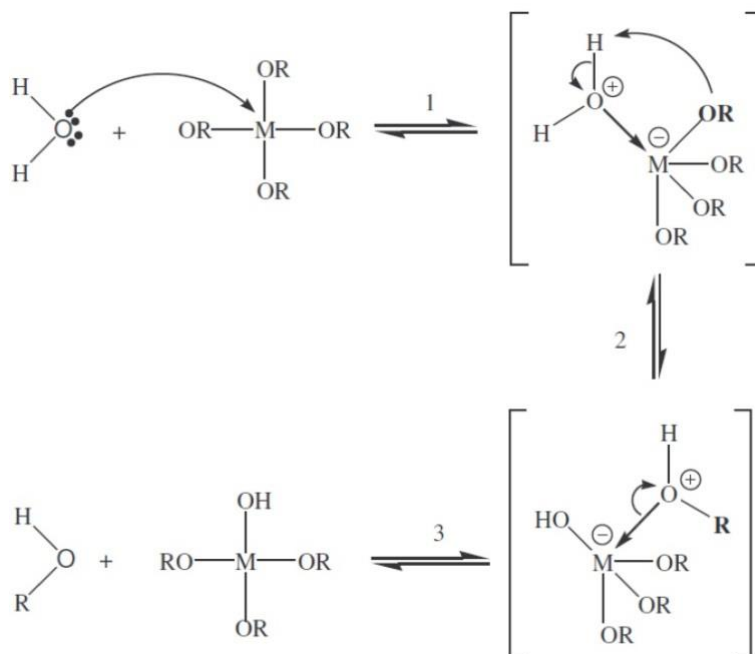


Figure 4: Hydrolysis mechanisms of metal alkoxides $M(OR)_n$
[23]

Condensation

After total or partial hydrolysis of alkoxides, those compounds can react with one another; through nucleophilic substitution (OH groups are good nucleophilic groups). It can as a result enable the growth of chains and then a tridimensional inorganic network via the formation of M-O-M bonds.

Such a type of propagation reaction is called condensation. This “polymerization process” quite often simultaneous with hydrolysis, can be complicated because several other mechanisms can compete. The relative importance of each mechanism depends on the experimental conditions. In the case of metallic alkoxides, two polycondensations are involved: alkoxolation and oxolation but will not be more detailed in this report.

Catalytic mechanisms

Acid catalysis: The steps of hydrolysis and condensation happen in acid catalysis through a nucleophilic SN2 substitution mechanism, after protonation of the alkoxide. Figure 5 shows the reaction mechanism of acid catalysis, which is valid for both steps (you can simply replace H₂O by SiOH to go from the hydrolysis to the condensation).

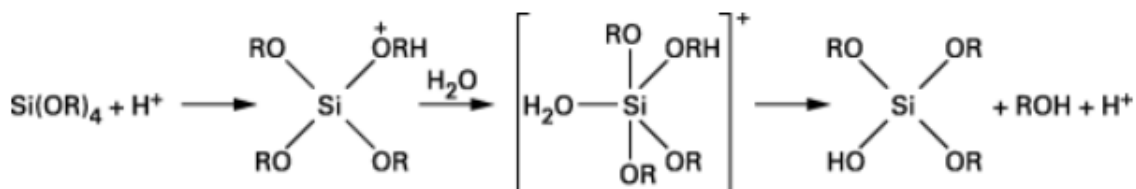


Figure 5 - Reaction mechanism of acid catalysis in the case of silicon alkoxides [23]

Base catalysis

The mechanism of base catalysis occurs following a slightly different reaction according to the use of a conventional base or fluoride ion. In the first case, there is an attack of the alkoxide by hydroxyl ion as a SN2 mechanism (figure 6), whereas in the second case, silicon derivate is first created followed by an addition of water and as a second step, elimination of water and a fluoride ion.

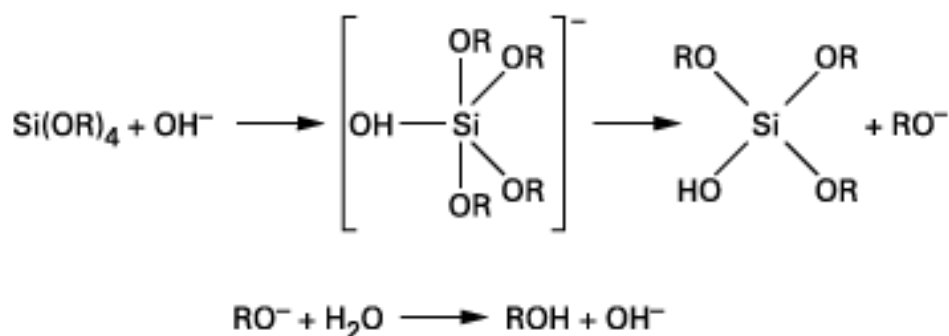


Figure 6 - Alkoxide attack by hydroxyl ion in a SN2 mechanism [23]

pH influence

As indicated above, the gel time strongly depends on the type of catalysis. We are able to see on Table 3, the time dependence of the gel according to the pH and the catalyst used.

Catalyst	pH of the solution	Gel time (h)
None	5	1000
HF	1.9	12
HCl	0	92
HNO ₃	0	100
H ₂ SO ₄	0	106
CH ₃ COOH	3.7	76
NH ₄ OH	10	107

Table 3 - pH and catalyst influence on the gel time [22]

The results show that the “counter ion” has not a significant importance in the case of strong acids, while the pH may be the only important parameter in the other cases. Some studies have been made about the kinetics of gelation as function of pH for various types of acid. The results show that there is an optimum pH of less than 1 or between 3 and 6, which probably corresponds to a compromise between protonation of the leaving group and protonation of the nucleophile.

An acidic pH catalyzes the hydrolysis while a basic pH catalyzes the condensation. A strong degree of hydrolysis (acidic pH) thus promotes the growth of the network and leads to a polymeric solution. Under acid catalysis the gel formed is called “polymeric gel” and we obtain after that a gelling open structure.

The reactivity of the precursor towards the hydrolysis decreases as it is hydrolyzed. The condensation step, which is kinetically slow, then begins to form chains, which afterward crosslink and form the network. The process leads to the formation of the gel.

On the other hand, a low rate of hydrolysis (basic pH) rather promotes nucleation and leads to forming a colloidal solution. In this case, we obtain morphology with spherical particles or block but brittle and opaque, and the pore size can be controlled (in opposition to acid catalysis). The gel formed is in this way called “colloidal gel” and has a large pore structure (clusters).

Solvent

In addition to the main alcohols (usually methanol or ethanol), several polar solvents (miscible with water) were used for the sol-gel polymerization: formamide, dimethylformamide DMF, tetrahydrofuran THF, dioxane... It is even possible to produce gels in hydrophobic solvents such as tributyl phosphate or dibutylformamide, on the condition that the hydrolysis releases enough alcohol to allow gelation; the residual alcohol is subsequently evaporated while standing at room temperature.

Metal alkoxide Precursors

$M(OR)_z$, which are called transition metal alkoxides, more specifically those of the d^0 transition metals (e.g. Ti, Zr), are widely used as molecular precursors to produce glasses and ceramics materials. Those alkoxides are usually quite reactive because of highly electronegative OR groups that stabilize M (in its highest oxidation state) and make it very susceptible to nucleophilic attack.

Several parameters make metal alkoxides behave differently from group IV silicon alkoxides ($Si(OR)_4$), which remains the most generally used precursors for the sol-gel method. We can isolate a few factors from a previous study [49]:

- Transition metals are less stable towards hydrolysis, condensation and any other nucleophilic reaction because they are precisely more electrophilic due to their lower electronegativity.
- The coordination of the metal alkoxides can be extended and often have several stable states of coordination.
- Its high reactivity requires a very precise control of moisture and hydrolysis conditions in order to form gels rather than precipitates.
- The study of the hydrolysis and condensation of transition metal alkoxides is more difficult than in the case of silicon alkoxides generally because of the rapid kinetics of the nucleophilic reactions.

Sol-gel process applied to our project

Preparing $\text{CaO-TiO}_2\text{-P}_2\text{O}_5$ glasses by sol-gel method has several convenient points compared to other preparation techniques. The process can be modified to produce porous materials which are a key factor in tissue engineering, where meso- and macrostructure are important in terms of cells growth. The low temperature of the procedure is also an interesting advantage for the application in drug delivery products, where the gel acts as a soluble matrix. Inclusion of a biocompatible polymer in the synthesis in order to produce composite materials with significant properties is also a promising challenge [20]. There are not much recent publications on the direct synthesis of $\text{CaO-TiO}_2\text{-P}_2\text{O}_5$ via the sol-gel method.

4.3.3.1. Precursors

The reason for the lack of publications concerning the preparation of phosphate based glasses via the sol-gel process is, according to a specific study [22], that it is more challenging and strict than the preparation of silicate based glasses by the same process. The basic issue concerns the right phosphorus precursor (hydrolysis of alkyl phosphates is very slow when proceeded via sol-gel), phosphate ions tend to form precipitates rather than network structure based upon P-O-P bonding [21].

This is why this project intent to find suitable precursors for the use via the sol-gel process with one specific purpose: to enhance angiogenesis in tissue regeneration through the formation of organometallic networks which must contain calcium and phosphate and

must deliver in an appropriate rate, but using non-toxic byproducts, as we will obtain organic/inorganic hybrid nanoparticles.

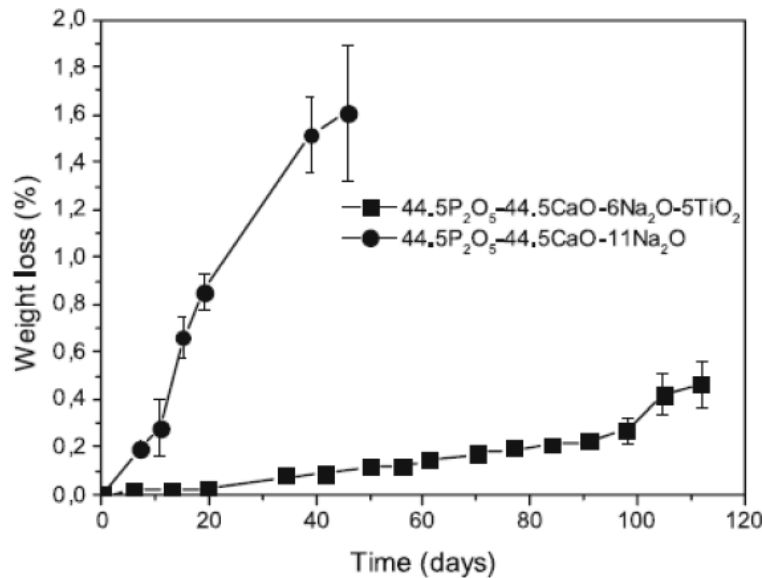
A major part of this project focuses on the use of phytic acid as the phosphorus precursor. Indeed, phytic acid was found to assist calcium being incorporated into glass network. In vitro tests in simulated body fluid were performed on a specific glass ($\text{CaO-SiO}_2\text{-P}_2\text{O}_5$) and the results have shown that this type of glass was bioactive over a much larger compositional range, and precisely at high phosphate content. Besides, the phytic acid itself is non-toxic.

4.3.4. Controlled release glasses (CRG)

Controlled release glasses are a type of materials with an attractive property; they can dissolve in aqueous media with no solid parts remaining. The degradation encountered is an erosion controlled process, the degradation rate being constant and independent of time and [25]. They can be designed into different forms such as tubes, fibers, powder, or monoliths.

Phosphate glasses have several main properties which support applications for biomaterials applications. These glasses containing ions commonly present in the human body (for example Ca^{2+} or Na^+) can be registered as biocompatible and bioresorbable materials. Studies have shown that it is possible to have a predictable dissolution rate which can be controlled by varying the composition (Figure 7). The degradation rate of sol-gel glasses aims to be adjustable from a few days, with high phosphate content, to a few years, with almost pure silicate; therefore it meets the requirements of different desired applications, such as controlled release and bone replacement. Also, by being completely amorphous, the dissolution appears to be rather uniform with only a little risk of residual fragments causing sterile inflammation. In our case and as already mentioned, $\text{CaO-TiO}_2\text{-P}_2\text{O}_5$ glasses tend to be very interesting for biomedical applications. These materials can actually be prepared in order to become bioresorbable, with a release of Ca^{2+} , which rightly appears to stimulate cell proliferation. The presence of the TiO_2 content is totally justified, as it allows reducing the dissolution rate and therefore the control of this rate. The TiO_2 has a small ionic radius, and the Ti^{4+} ion is precisely tetravalent, meaning it is able to penetrate into the structure and thereby generate a high degree of crosslinking of the network. But TiO_2 not only appears to be a major importance in the dissolution and control rate but also causes to have significant effects on the gene up-regulation, according to recent study [26].

Besides, it has been reported that the impact of the ionic environment generated by the release of ions from phosphate based glasses can be extended to the phosphate ions as well, as it acts as an extracellular matrix responsible for the release of Cbfa-1, an important bone marker, from bone cells [27].



Weight loss versus dissolution time for two different CaP glasses during degradation in SBF at 37 °C. Error bars not shown if smaller than symbols

Figure 7 - Weight loss vs degradation for 2 bioactive glasses) [26]

4.3.5. Impact on biocompatibility – Cytotoxicity of byproducts

Because the aim of this project is to study particles obtained by two major precursors, which are supposed to then embedded in a polymeric matrix, we have to study the consider the biodegradability of the whole composite. The matrix in question is considered to be PLA (polylactic acid), for its biodegradable behavior. As previous studies carried out at IBEC [28], we want to obtain a fibrous scaffold through electrospinning with embedded phosphate based glasses in order to mimic an extracellular matrix.

The degradation of the scaffold will start the liberation of the Ca²⁺ ions from the nanoparticles, which undoubtedly generate liberation of byproducts used during the

synthesis as well. The utilization of precursors whose byproducts should be biocompatible and biodegradable are required, the body should be able to eliminate them afterwards.

The precursors involved so far in the synthesis of G5 nanoparticles were synthesized using whether absolute ethanol or 2-methoxyethanol, which are known as cytotoxic when liberated in the body. Our study focuses then on the organic byproducts released by the two main precursors that we used: calcium propionate and phytic acid.

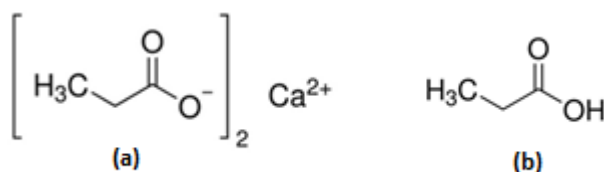


Figure 9 - calcium propionate salt (a) and its propionic acid (b) formula (from Sigma-Aldrich)

The use of calcium propionate salt (Figure 9-a) will lead to the liberation of both Ca²⁺ ions and the associated propionic acid (Figure 9-b). Previous studies on propionic acid toxicity have been conducted [29], and have demonstrated that propionic acid is a normal metabolite in the human body, since it is utilized by most organs and tissues. Propionic acid is metabolized in human body in a similar way to that of fatty acids [30]. The presence of propionic acid as byproducts of the precursors used to synthesize particles is therefore not a problem.

The other precursor used is the phytic acid solution (50% w/w H₂O) as described in Figure 10.

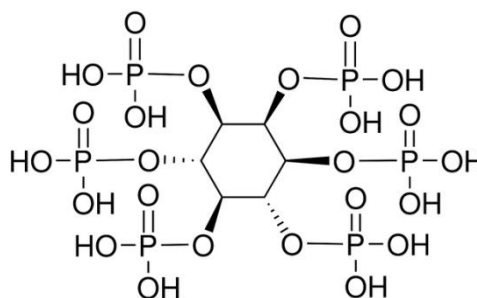


Figure 10 - Phytic acid solution (50% w/w H₂O) (from Sigma Aldrich)

Phytic acid has not been used in too many ways as a precursor for sol-gel synthesis and when it did, it was essentially to produce silicon based particles, meaning that the organic part was removed after calcination. What we intend to do (using the phytic acid to get organic/inorganic hybrid particles) has no significant previous work to be referred to. However we can base our study about toxicity of this precursor on the literature, in which phytic acid has appeared to be non-toxic for biomedical applications [31], [32]. Besides, the fact that phytic acid has really good affinity with multivalent metal ions, such as calcium for example, makes it an interesting precursor as it can promotes calcium incorporation into the network at relatively low temperatures (ideal for sol-gel process). The phytic acid use actually has another advantage: it has a “carrier” behavior. It means that it can be able to carry one compound, transport it and then release it, which appears to be a very interesting feature for our desired controlled glasses release applications.

4.4. Objectives

The aim of this project is essentially developing new a biocompatible and bioresorbable material, which would be able to release Ca^{2+} at an appropriate rate in the damaged area of bone or skin in order to stimulate and promote quick and better tissue regeneration. This project focuses on the development and fabrication of new bioactive glass nanoparticles using different precursors by the wet methods. The purpose of those particles is to be embedded later in a polymer matrix through electrospinning.

- Synthesis and understanding of precursors impact of hybrid nanoparticles with bioactive properties.
- Characterization of the particles
- Evaluation of the biological response to different types of obtained particles on mesenchymal cells.

5. Materials and methods

5.1. Synthesis of the glass nanoparticles

Two main different types of glass nanoparticles were synthesized by the sol-gel method during this project. The first synthesis was performed to produce calcium-phosphate based glasses known as G5 (Glass composition are identified as GX (G standing for Glass, and X corresponding to the atomic percentage of titanium in the glass composition), including the following compounds: CaO, P₂O₅, Na₂O and TiO₂, with a molar ratio of respectively 44,5 CaO - 44,5 P₂O₅ – 6 Na₂O – 5 TiO₂.

G5 glass nanoparticles studies have been rather promising indeed. It has been shown that G5 features the best compromise between the degradation rate and the amount of Titanium involved [33].

Later on, the major task of this project is related on the synthesis of other G5 nanoparticles, using different new precursors and trying to grasp the understanding of the different synthesis parameters, still via the same sol-gel process but with only three alkoxides precursors, the Na₂O precursor being removed. The chemical composition became then: 47.5 CaO - 47.5 P₂O₅ – 5 TiO₂. The challenging part of this project is that it is the first time that we will produce organic/inorganic hybrid nanoparticles of this type using these precursors, and therefore sees the impact of the remaining organic part as byproducts.

Synthesis of the precursors

To achieve these synthesis, and as already mentioned, we use alkoxide precursors for their good reactivity in the sol-gel process mechanisms. The following precursors are initially obtained according to the protocol outlined in previous studies (some alkoxides precursors have been prepared by us, the others by the previous or current working team):

- CaO alkoxide precursor is prepared using metallic calcium and 2-methoxyethanol, under inert atmosphere (reaction at a temperature of 124°C for 24h). We obtain a solution of calcium methoxyethoxide with a calcium concentration of [Ca²⁺] = 1mol/L.

- P_2O_5 alkoxides precursor is obtained by refluxing pure phosphorus pentoxide in absolute ethanol at 78°C. The process needs to be performed under inert atmosphere. The alkoxide precursor solution has a concentration in $[P_2O_5]$ of 2 mol/L.
- TiO_2 alkoxide precursor is prepared by the dilution of a Titanium (IV) isopropoxide (95%) in absolute ethanol to obtain a final 2mol/L concentration of $[TiO^{4+}]$ in solution.
- Na_2O (only used for the first synthesis) is obtained by refluxing metallic sodium in 2-methoxyethanol under inert atmosphere for 24h at 124°C. The final sodium methoxyethoxide has a concentration in $[Na^{2+}]$ of 2mol/L.

In order for our desired phosphate-based glass to meet the requirements previously requested, that is to say molar ratio, calculi have been carried out. Molar ratios are exposed in Table 4 and Table 5:

Precursor	Theoretical molar ratio %	Concentration in mol/L
CaO	44,5	0.92
P_2O_5	44,5	2
TiO_2	5	1.95
Na_2O	6	1.99

Table 4 - First sol-gel synthesis including Na precursor

Precursor	Theoretical molar ratio %	Concentration in mol/L
CaO	47,5	0.92
P_2O_5	47,5	2
TiO_2	5	1.95

Table 5 - Major synthesis of the phosphate-based glass
desired (5% TiO_2 without Na)

First case – G5 nanoparticles preparation with Na

According to the desired composition (see Table 6), the synthesis begins with the preparation of a precursor solution in an inert atmosphere. We are using a hermetically sealed balloon flask because such precursors are unstable in the presence of water, and quite reactive (this is why we inject each solution using syringes through the membrane). The different amounts of each precursor, previously calculated, are introduced according to a special order and procedure. We first took the calcium alkoxide precursor solution as a reference and adjusted our volumes in order for the synthesis to have a final ratio of 5% precursor volume and 95% for the solvent. For this first experiment, we used only precursors already prepared by the laboratory team. Details of the calculi are available in the diary book of the laboratory.

Precursor	Theoretical molar ratio %	Concentration in mol/L	Volume in mL
CaO	44,5	0,92	6,13
P₂O₅	44,5	2	2,81
TiO₂	5	1,95	0,32
Na₂O	6	1,99	0,76

Table 6 - Concentrations and volumes of precursors for the first synthesis

We used in that case 10mL in total for the precursors' solution, which appears to be an amount that we managed to reduce in order to work with basically 5mL for the following preparations.

First, the precursors' solution is carried out by mixing respectively the calcium, sodium and titanium precursors stirred during 1h (under argon inert atmosphere) in order to obtain a homogeneous mixture. Subsequently and according to the 5%-95% ratio of the alkoxide/solvent volumes, we add the 1,4-dioxane, a suitable nonpolar organic solvent which does not dissolve the newly formed nanoparticles [28]. Then the phosphorus precursor is added "a drop at a time" using a syringe and a pump that will control the output

rate of the solution at an approximately rate of 1mL/h, the flask being cool down in water and ice-based bath to avoid undesired exothermic reactions.

The hydrolysis and condensations reactions which allow the gel to form are accelerated by introducing an aqueous catalyst in the balloon flask. We used a basic catalyst because it enables the formation of short, highly branched chains and favors the condensation kinetics [34]. The catalyst solution includes water, ammoniac and ethanol with a desired ratio of 60moles of H_2O /0,3moles NH_3 / 12 moles of ethanol for 1mole of titanium used in the precursor solution. Because in our preparation we only use $0,624 \cdot 10^{-3}$ moles of titanium, we had to adapt our catalyst.

The water amount attempts to enhance the hydrolysis which in our case helps to control the balance with the condensation reaction (actually supported by the basicity of the whole solution).

As a final step, we transfer our solution into a glass bottle and let the mixture under stirring at a temperature of 75/80°C for 4 days. We can observe a change in the color of the solution, which firstly appears to be yellow orange and turn into white after 48-72h. This effect is expected as it shows the “gelation process”, production of the nanoparticles separated from the liquid phase.

To extract our powder from the liquid phase, we performed centrifugations:

- First centrifugation at 3000 rpm for 10 minutes at 4°C - separate the liquid phase (dioxane solvent) from the particles.
- Addition of ethanol to “wash” the particles
- Five repeated “washing” centrifugations
- Put the centrifugation tubes with the particles collected in the stove at 80°C – drying process.

The powder obtained is then supposed to be mixed with a biodegradable polymer solution (PLA, gelatin) in order to be processed into fibers by electrospinning for example.

Second case – G5 nanoparticles preparation without Na (Main part of the project)

In this case, the mechanisms involved are the same as in the previous one, and the desired morphology of the particles stays the same as well. However, the glasses produced this time have some different compositions. Indeed compounds of calcium (Ca), and phosphate (P) have been synthesized through various alkoxide precursors in order to test their respective influence on the hydrolysis and therefore on the final composition and morphology of the oxide.

We performed several syntheses, involving the following parameters:

- Concentrations
- Temperature
- Time
- Ratio precursors/solvent

Precursors used:

As calcium (Ca) precursors		
Calcium oxide CaO	Calcium propionate $C_6H_{10}CaO_4$	D-Pantothenic acid hemicalcium salt $C_{18}H_{32}CaN_2O_{10}$

- ❖ Calcium oxide CaO: Same preparation as mentioned before in the first experiment.
- ❖ Calcium propionate $C_6H_{10}CaO_4$ (Figure 11): We used it as dry powder (97% pure) solubilized in propionic acid under inert atmosphere at the very beginning of the synthesis. Stirring for 1h to dissolve it as much as possible before introducing other precursors. We weighted (according to the calculi) 0,6589g of calcium propionate and put it inside the balloon flask and then we added the propionic acid inside under stirring. We waited 1h after injection of the titanium precursor.

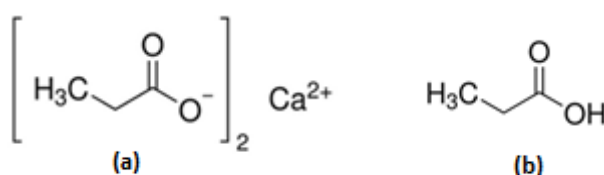


Figure 11 - calcium propionate salt (a) and its propionic acid (b) formula
(from Sigma-Aldrich)

- ❖ Calcium D-pantothenate salt $C_{18}H_{32}CaN_2O_{10}$ (Figure 12): It is a white crystalline powder associated to the pantothenic acid, which is better known as vitamin B5. It is slightly soluble in alcohol but easily in water, as it is a hygroscopic compound. D-pantothenic acid may have a role in controlling keratinocyte proliferation and differentiation. According to other current studies, a dissolution using the trifluoroethanol (TFE) could be a good option but has to be very carefully prepared (40min at 40°C), with a precise control of the temperature. Otherwise and in our case, the dissolution was directly followed by solidification.

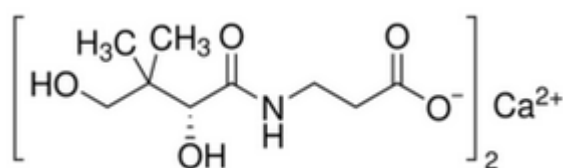


Figure 12 - D-Pantothenic acid hemicalcium salt formula (from Sigma Aldrich)

As phosphorus (P) precursors		
Phosphorus pentoxide P_2O_5		Phytic acid $C_6H_{18}O_{24}P_6$ (in 50% water solution and in salt powder)

- ❖ Phosphorus pentoxide P_2O_5 : Same preparation as mentioned before in the first experiment.
- ❖ Phytic acid $C_6H_{18}O_{24}P_6$ in 50% water solution: This precursor is already prepared by Sigma Aldrich in a 205mL glass bottle. We introduced the 1,04mL “drop by drop” using the pump at a rate of 1mL/h; important precipitation occurs quite quickly.
- ❖ Phytic acid sodium salt hydrate $C_6H_{18}O_{24}P_6$: It is a very fine white powder. After several researches and tests in order to dissolve it (water, Phosphate buffered saline PBS, and temperature control), we were unable to find a good procedure to use it as an alternative precursor compared to the one at 50% water.

The titanium (Ti) precursors used in the following preparation is the same as in the previous experiments: Titanium dioxide TiO_2 . As seen on the previous projects, the amount of titanium is the content that critically affects the satiability of the glass network, and for that actually modifies the rate of degradation of the glass. After past experiences on different compositions for the titanium amount [34], it has been experimented that a 5%-titanium ratio is preferred. We then decided to keep this direction and make a 5%-titanium amount glass adjusting the other compounds for all our experiments (Table 7):

Precursor	Theoretical molar ratio %
CaO	47,5
P₂O₅	47,5
TiO₂	5

Table 7 - Composition molar ratio theoretical

The syntheses were performed following to the same procedure, which was obviously adapted according to the specifics of each “new tested” precursor.

- Injection of the precursors of calcium, titanium, and then phosphorus “drop by drop” (which is the one who initiates the hydrolysis)
- We let the solution under stirring and water and ice bath until complete hydrolysis, when the solution turns into white.
- Centrifugation to separate the particles from the solvent and then five times more to “wash” them using ethanol. In those cases, we programmed the centrifuge for 10 minutes at 10000rpm or even 15000rpm in order to properly separate the nanoparticles.
- Put the centrifuge tubes in the stove at 80°C for approximately 4 to 6 hours.
- Grind the powder to remove agglomerations and to homogenize the particles.

As it has been mentioned before, we tried to adapt the preparation according to our different precursors, especially because we have been using both solid salt powder precursors as well as solution (more or less effective depending on solubility of the products selected).

5.2. Characterization methods

The first characterization method that we used was to show the morphology and the chemical composition of the particles.

5.2.1. Scanning Electron Microscopy (SEM)

The scanning electron microscopy SEM is a powerful observation of surface topography technique. It is mainly based on the detection of secondary electrons emerging from the surface under the impact of a very fine brush of primary electrons sweeping the observed surface and provides images with a resolving power often less than 5 nm and a great depth of field.

The SEM uses, in addition, the others interactions of the primary electrons with the sample: emergence of the backscattered electrons, absorption of the primary electrons, photon X emission, and sometimes the interaction with photons which are close to the visible spectrum. Each of these interactions often gives us significant informations of the topography and/ or the surface composition.

This technique is used to form an almost parallel brush, fine (up to few nanometers), strongly accelerated by adjustable voltages from 0.1 to 30 kV, to focus on the area to be examined and to scan it gradually. Suitable detectors, specific electrons detectors (secondary, backscattered, sometimes absorbed...), supplemented by photon detectors allow to gather significant signals when scanning the surface and to form several meaningful images.

In our case, Scanning Electron Microscopy and Energy Dispersive Spectrometry analysis we performed on a Quanta 200 scanning electron microscope equipped with an EDX energy dispersive spectrometer. Working under high vacuum and high voltage, the samples had to be covered with a layer of graphite to prevent negative charges from accumulating in the surface of the material, which could then deforms the electron beam and alters its effective energy. The energy dispersive spectrometry allows us to know the chemical composition of the samples.

To prepare our powder samples, we disperse a very small amount of the nanoparticles on the sample holder (which has been divided on 4 different parts). Below (Image 1) is presented an example of graphite sample holder with powder nanoparticles.



Image 1 - Sample holder covered with graphite / ready for SEM analysis

Scanning electron microscope field emission (FE-SEM)

We performed our observations on a FEI Nova NanoSEM. With this equipment, unlike the SEM, we were able to work under low vacuum system, which means using lower voltage (the graphite coating is not necessary in this case). Thus, we have achieved observations of our powders with higher magnifications.

5.2.2. Degradation

One of the important parameter of our phosphate-based glasses is absorbability. In order to control and to predict our powder behavior “in the body” conditions, we have to study the degradation rate. Indeed the release of calcium, phosphorus and sodium is studied so we can know how they behave (how fast and in which amount they degrade in the body). Previous studies have shown that calcium have a key role in blood vessels formation, thanks to its angiogenic properties. It is then quite important to have a good knowledge on how it is released.

To do so we carried out two important assays: Ca release and pH tests.

These tests require a special procedure. For the measurements to be relevant, in agreement with our “in the body” conditions, we prepared our samples and let them in the sterilizer at 37, 5°C during the whole time of the assays.

We used well-plate to prepare our powder samples. After having selected three different nanoparticles powders, based on their shape and chemical composition, we placed them as described below (Figure 13):

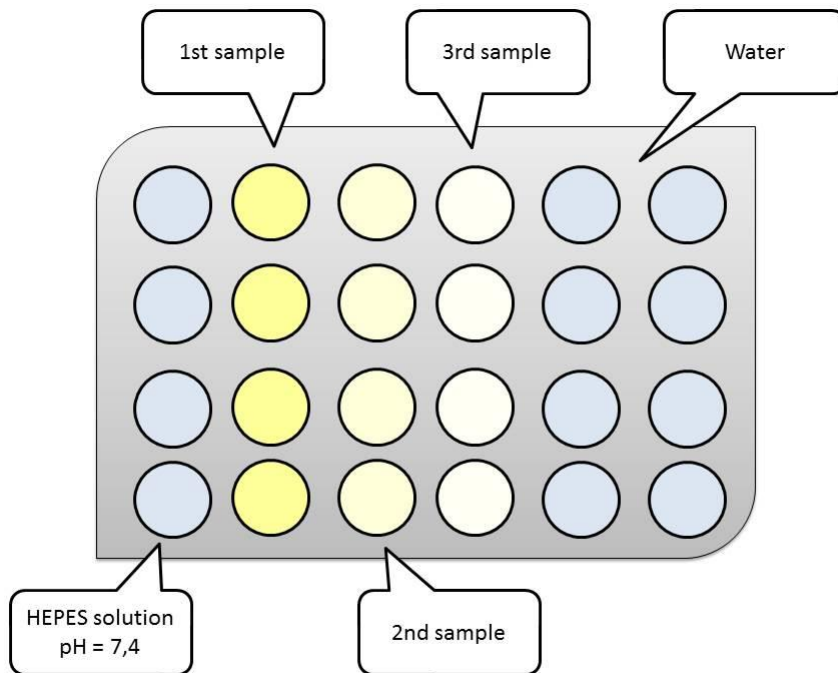


Figure 13 - Preparation of nanoparticles powder for pH and Ca release assays

We put on each slot, 15 micrograms of powder, covered by a cellulose acetate filter and a Teflon® ring (so that it keeps the filter from moving and the particles from going through). Therefore we submerged each sample with 750 microliters of a HEPES solution with a pH of 7.4 to stay under conditions close to reality. (Figure 14)

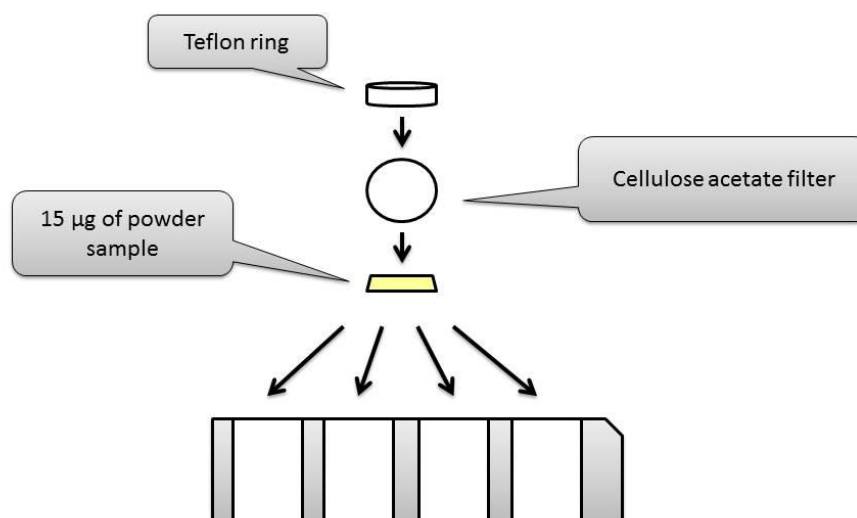


Figure 14 - Components of assays preparation

5.2.2.1. Ca^{2+} release

The equipment used is a pH & ion meter GLP22+ as shown in the next figure. The red electrode is used to control the pH of our solutions and the green one to measure the evolution of conductivity of free ions, in our case Ca^{2+} , and then give us directly the concentration of ions. This involves first a calibration of the engine; we measure the conductivity of five solutions containing five different calcium concentrations; 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} and 10^{-1} mol/L. With this calibration, we obtain the logarithmic ratio between concentration and conductivity after plotting the following curve line (Figure 15).

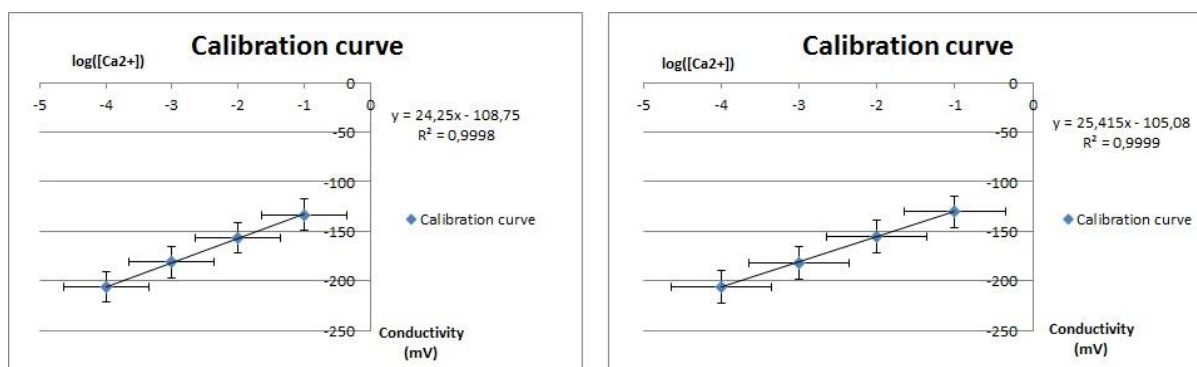


Figure 15 - Calibration curves for the two calcium release tests performed

The equation relied to our relation between concentration of calcium in mol/L and conductivity in mV is (Equation 1 and 2):

Test 1

$$\text{Conductivity} = 24.25 \cdot \log [\text{Ca}^{2+}] - 108.75$$

$$\text{This is equal to: } [\text{Ca}^{2+}] = 10^{((\text{conductivity} + 108.75)/24.25)} \quad (1)$$

Test 2

$$\text{Conductivity} = 24.415 \cdot \log [\text{Ca}^{2+}] - 105.08$$

$$\text{This is equal to: } [\text{Ca}^{2+}] = 10^{((\text{conductivity} + 105.08)/24.415)} \quad (2)$$

5.2.2.2. pH measurements

pH measurements were carried out to measure the pH increase of our HEPES solution. HEPES is especially used to work with cells as it is much better at maintaining physiological pH than usual bicarbonate buffers for cell culture. The pH increase of our system is due to the nanoparticles ion release.

The measurement is performed on the same Crison equipment as mentioned before, using the red electrode. We measure the pH of each solution transferred in several Eppendorf tubes, carefully washing the electrode after each measurements not to affect other results.

5.2.3. X-Ray Diffraction

Another important aspect of the glass structure in which we are interested is the crystallinity. As we already mentioned before, the degradability of glass depends on its crystallinity. Indeed the more crystalline is the material structure; the lower is the rate of degradation. This is why we prepared a few samples of our powders to be analyzed. In this regard, we performed diffraction analysis (DRX) on a PAN-alytical's X'Pert PRO system. Because we are actually handling “glasses”, crystalline structures are far from expected.

Unfortunately the selection process of which sample we chose for the X-ray diffraction was not based on their properties, good composition or morphology but on the amount of powders that is needed to carry out such characterization method.

Here are the powders selected (for their relative good amount) to go under examination:

- G5/1mL Phytic Acid/5-95%
- G5/1mL Phytic Acid/20-80% (07/10)
- G5/1mL Phytic Acid/2,5-97,5%
- G5/0,1mL Phytic Acid/20-80%
- G5/44,5 CaO - 44,5 P₂O₅ – 6 Na₂O – 5 TiO₂/20-80%

In order to analyze the results, we are using a database that gathers together the diffraction peaks of all the crystalline phases which would be encountered.

5.2.4. Zeta potential and size measurements of particles

In order to perform these series of assays, we used a Zetasizer equipment (Zetasizer Nano S) (Image 2):



Image 2 - Zetasizer and software associated

The zeta potential is an important parameter characteristic of electrical properties of solid/liquid or liquid/gaseous interfaces. It is a function of the surface charge of the particle, any adsorbed layer at the interface, as well as the composition of the medium surrounding it. It actually reflects the effective charge on the particle and is, in consequence related to the electrostatic repulsion between them.

Nanoparticles have a surface charge that is able to attract a thin layer of ions from opposite charge to their surface. This layer of ions “travels” with the nanoparticle as it diffuses throughout the solution (Figure 16 - 17 [36]). The electrical potential at the boundary of the double layer is known as the Zeta potential of the particles, this value usually predict the colloidal stability. The range of values is commonly from -100mV to +100mV. Basically, if the zeta potential is higher than +20/25 mV or less than -20/25 mV, therefore the nanoparticles have good degree of stability.

We took the very same five powder samples that we used for the X-Ray diffraction for our Zeta potential assays (see X-ray part).

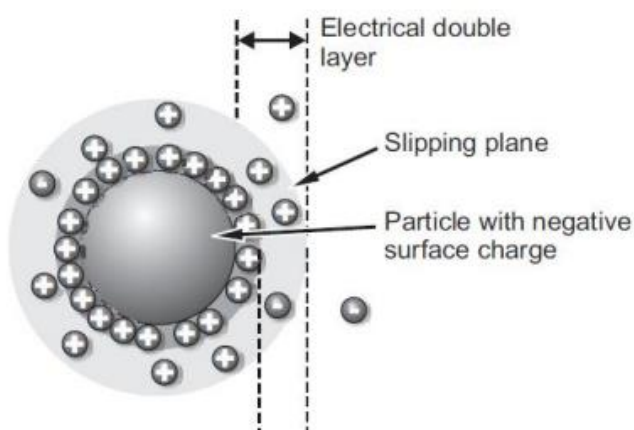


Figure 16 - Electric double layer surrounding [36]

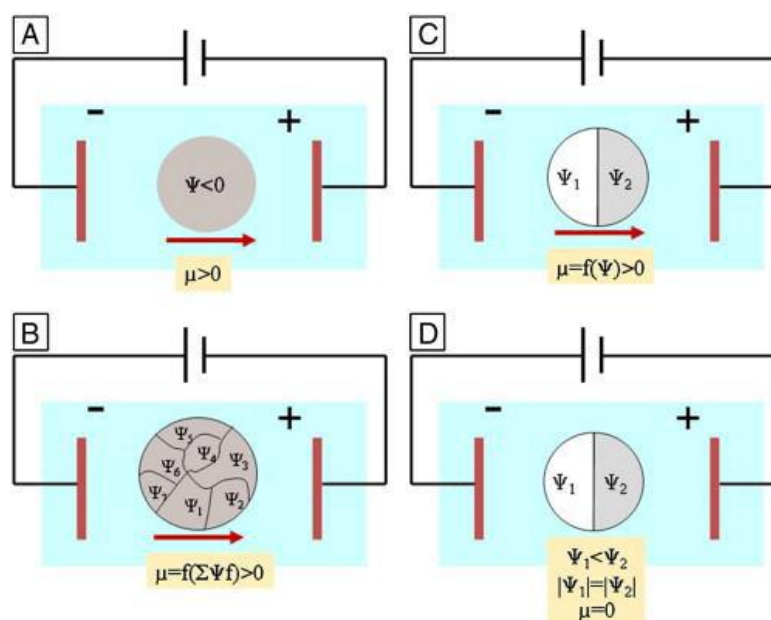


Figure 17 - Surface charge distribution [51]

5.3. Cell culture

Finally, at the very end of this project, we tried to evaluate the performance of our nanoparticles with cells. As the final purpose is for the particles to be used as scaffolds or delivering agents integrated in scaffolds, it should promote osteogenesis and/or angiogenesis. The cells involved in this process should then have a tendency to proliferate.

The cells that we used for this experiment were rat mesenchymal cells (rMSCs), which derive from bone marrow, where chosen due to their active work in skin and bone wound healing [37].

This type of cells have shown good behaviors in previous studies according to the fact that they are able to differentiate themselves into osteoblasts, chondroblasts, as well as keratinocytes and bone marrow stromal fibroblasts in vitro [38].

5.3.1. Cell seeding

As we already mentioned before, the preparation of our samples for the cell seeding follows the same procedure as for the pH and calcium release assays.

We used well-plate to prepare our powder samples. After having selected three different nanoparticles powders, based on their shape and chemical composition, we placed them as described below (Figure 18):

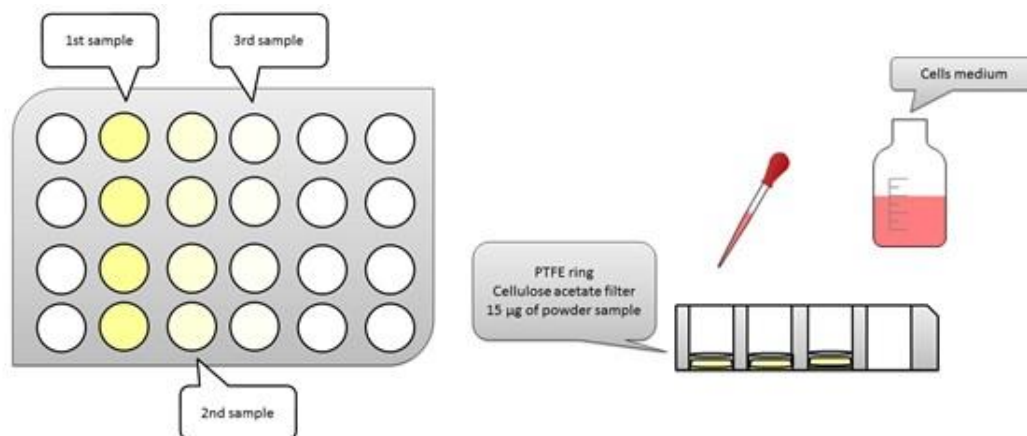


Figure 18 - Preparation of the powders for medium impregnation

- 1st sample: *G5 nanoparticles – 0,01mL phytic acid*
- 2nd sample: *G5 nanoparticles – 5/95% ratio phytic acid*
- 3rd sample: *G5 nanoparticles – first experiment with sodium (Na)*

After having placed our respective powder in the well-plate, we added a cellulose acetate filter and a polytetrafluoroethylene (PTFE) ring (previously sterilized using ethanol) so that the particles don't blend themselves with the cell culture medium.

Another well-plate prepared with cells is ready to receive the impregnated medium. We add the 500 μL of medium on each samples, 500 μL being the usual amount of medium that covers the powder-filter-ring system. In the same time, we “re-add” normal medium to the powder samples well-plate in order to use it the next day.

5.3.2. AlamarBlue® - Proliferation tests

We then decided to use a method to evaluate the evolution of the cell population; we selected the test with the AlamarBlue®.

The AlamarBlue® assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Basically, the system incorporates an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth [39]. The cells convert resazurin, which is blue, into reduced resorufin which is pink/red fluorescent. (Figure 19 [40])

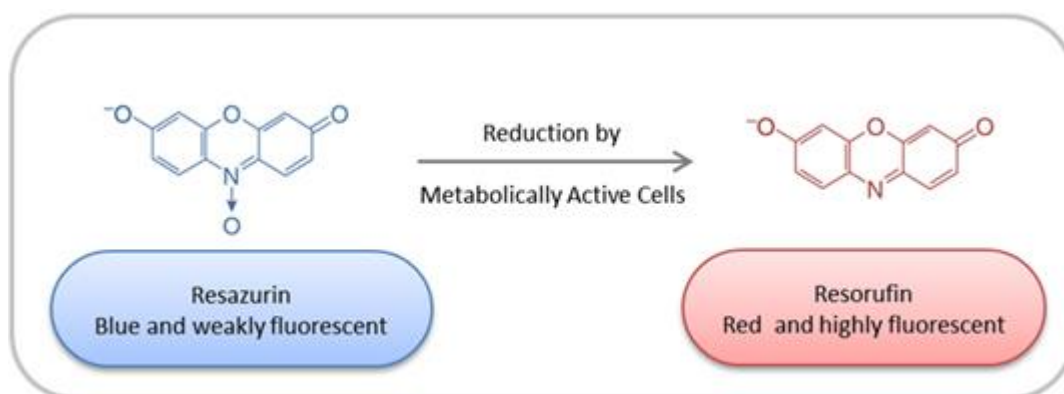


Figure 19 - Reduction process and colour change - Resazurin to resorufin [40]

These assays only let us take into account living cells, because non-viable or dead cells have a lower metabolic activity. Viable cells keep on converting resazurin into resorufin, in this way they generate a quantitative measure of viability and cytotoxicity. We use a pattern that allows us to evaluate this relationship, deducing the evolution of the number of cells in the medium by fluorescence measurements; it requires a calibration curve at the beginning of each test with the samples that are going to be used in the experiment (Figure 20).

The calibration curve is performed using a 24 well-plate. After having calculated the data required, we proceeded to the preparation of the experiment, the curve is obtained by

dilution from the highest number of cells. We used respectively 80.000/40.000/20.000/10.000/5.000/2.500/1.125/0 cells per well (approximately 500 µL), as described in the following scheme (Figure 21).

The calibration well-plate was incubated for 3 hours. The next step is the addition of AlamarBlue® solution, starting with a 50 µL removal of the incubation medium, and then addition of 50 µL of AlamarBlue® in a dark environment, so that the samples are partially protected from direct light. After three hours and thirty minutes of incubation, we put three volumes of each well into another 96 well-plate.

The results are given by a micro-plate reader.

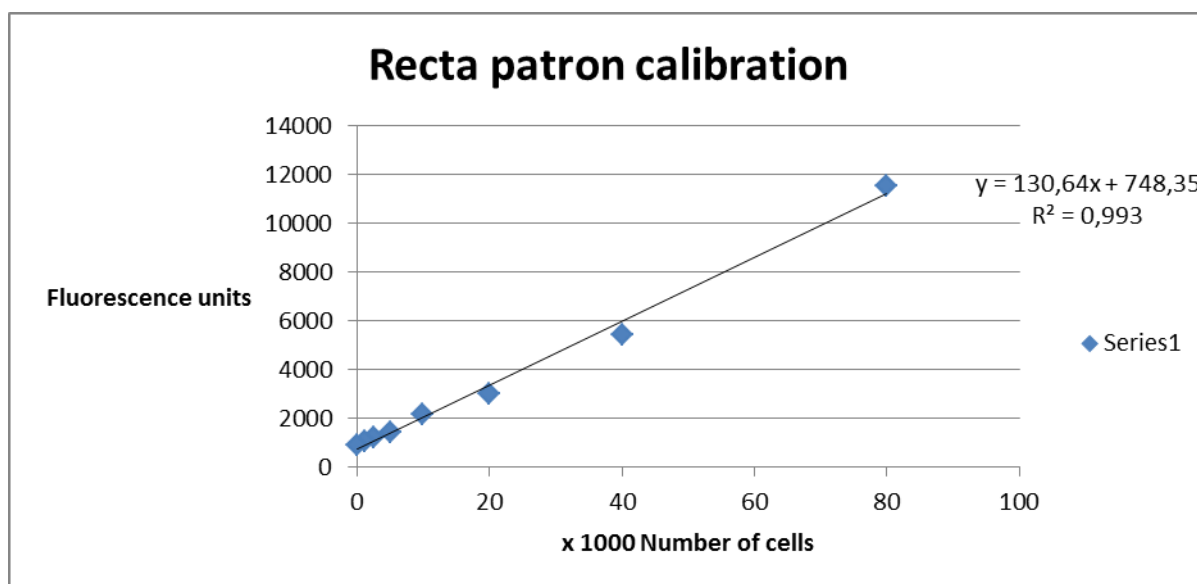


Figure 20 - Calibration curve recta patron

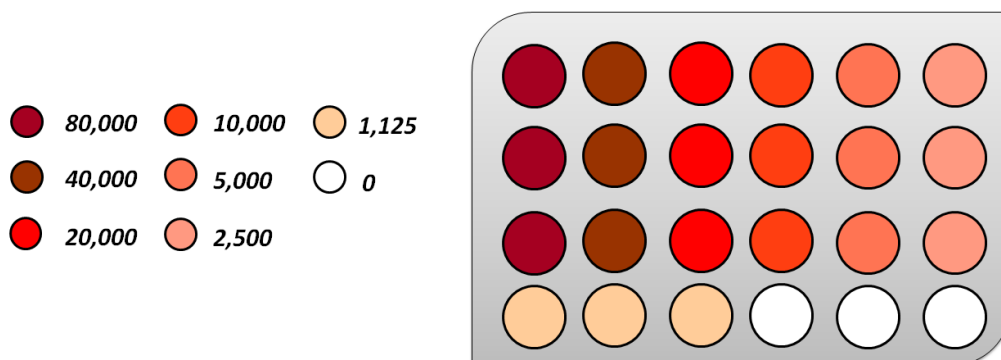


Figure 21 - number of cells par well

6. Results and Interpretations

6.1. Chemical Composition

As mentioned before, the aim was to achieve a final compound with a controlled release rate. In order to do so, several parameters are required, of which composition is a major part. 47.5 CaO - 47.5 P₂O₅ – 5 TiO₂ was the desired composition for our bioactive glass nanoparticles without Na. The following compositions of the different nanoparticles synthesis were obtained through the EDX energy dispersive spectrometry during the SEM sessions (Figure 22).

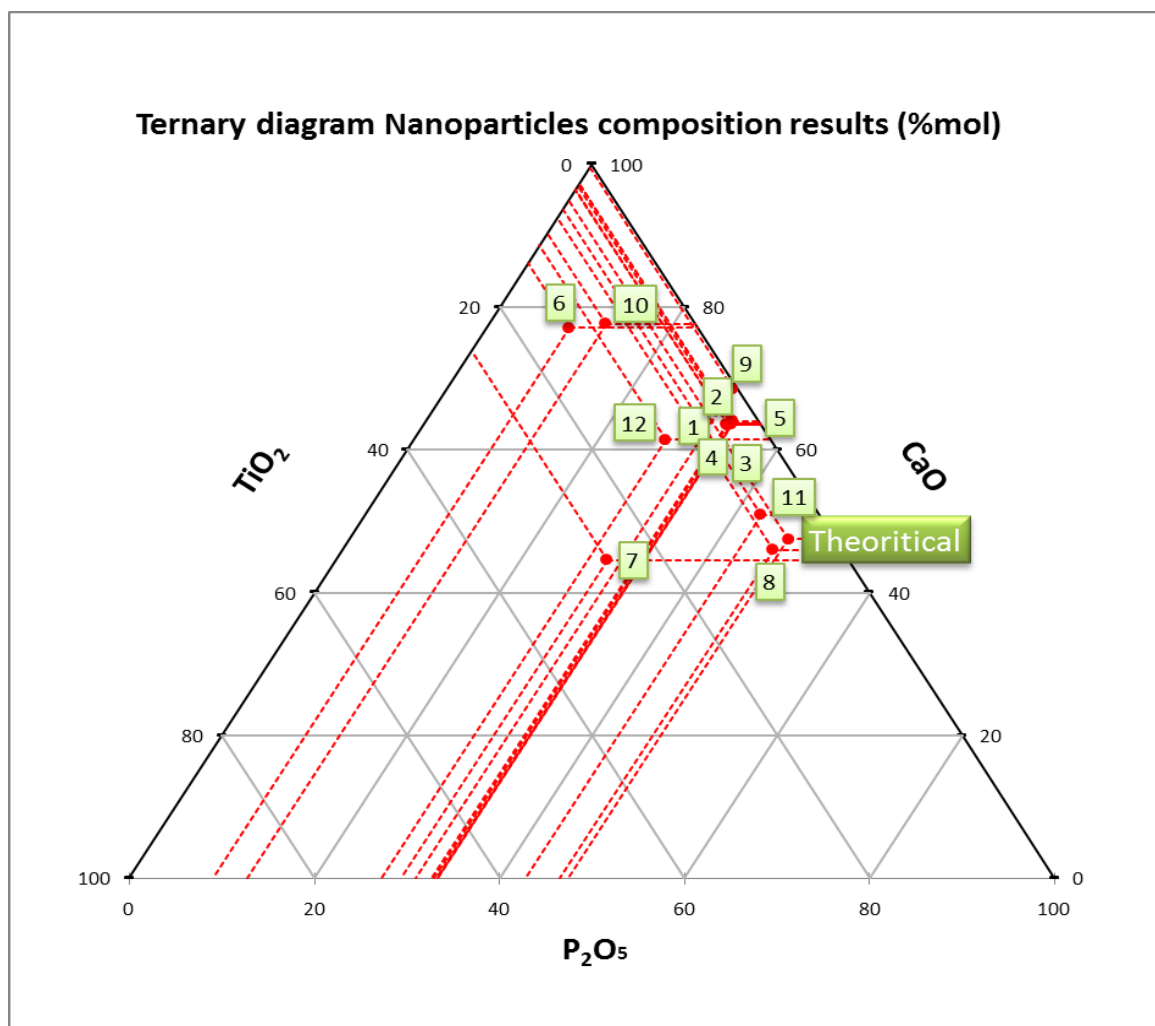


Figure 22 - Composition results of nanoparticles synthesized

Number	Type of nanoparticles	Composition %molar ratio obtained		
		P ₂ O ₅	CaO	TiO ₂
1	G5 Ca Pro 12h	32,91	63,49	3,6
2	G5 Ca Pro 6h	33,17	64,05	2,78
3	G5 Ca Pro 24h	33,28	63,93	2,79
4	G5 Ca Pro (0,6589*2g)	32,72	63,7	3,58
5	G5 Ca Pro 30°C	33,29	63,59	3,12
6	G5 Ca Pro 40°C	8,99	77,15	13,86
7	G5 Ca Pro 5/95	29,3	44,6	26,1
8	G5 Ac.Fit 07/10/13 (1,04ml)	46,57	46,01	7,42
9	G5 Ac.Fit (0,1ml)	30,92	68,61	0,47
10	G5 Ac.Fit (0,01ml)	12,7	77,65	9,65
11	G5 Ac.Fit (1,04ml) 5/95	42,8	50,9	6,3
12	G5 Ac.Fit (1,04ml) 2,5/97,5	27,23	61,42	11,35

Table 8 - Contents table of nanoparticles obtained/ composition %molar ratio
(for details of each samples – see next tables 9 and 10)

According to both the table 8 and the previous diagram, we can first say that the dispersion of the data show the difficulty of preventing sol-gel results with different precursors. Even tough, careful calculi have been carried out; the slightest change in one parameter of the synthesis can demonstrate its tendency to completely affect the final composition.

However, we are able to distinguish at least a tendency depending on whether we used the calcium propionate or the phytic acid precursors. As we can see on the diagram, the final composition of particles, when using the calcium propionate precursor, seems to be quite centralized with very close molar ratio difference between one another. But modifications have to be applied, in the concentrations and volumes studies, to obtain a higher amount of P₂O₅ and adjust CaO and TiO₂ levels.

On the other hand, the phytic acid precursor has shown much broader range of compositions. After having performed the first synthesis using the phytic acid, during which an important precipitation has been observed. Without studying the composition with the EDX spectrometer, further syntheses were carried out trying to reduce this precipitation phenomenon. Looking at the results it was obviously a mistake, as the molar ratio for P₂O₅

clearly decreases, which is quite logical according to the dilution applied to the phytic acid precursor.

After a review of the previous data, it appears we have at least two final nanoparticles with a relatively good composition, as the difference between the results and the theoretical desired composition:

- ✓ **G5 Ac.Fit 07/10/13 (1,04ml)**
- ✓ **G5 Ac.Fit (1,04ml) 5/95**

The 2 following tables (9-10) show the different procedures and results obtained:

	Concentrations	Volumes	Aging time	Temperature	Proportion precursors/solvent	%molar theoretical	%mol obtained
G5 Ca Pro 12h	0,6589g de Calcium Propionate (Ca(C ₂ H ₅ COO) ₂), [P2O5] = 2M, [TiO2] = 1,95M, Acid propionic 99%	3,22 ml d'acid propionic, 0,177ml de Ti, 20 ml de Dioxane, 1,61 ml de P2O5 (d.b.d)	12h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P2O5 - 5 TiO2	63,48CaO - 32,91P2O5 - 3,6 TiO2
G5 Ca Pro 6h	0,6589g de Calcium Propionate (Ca(C ₂ H ₅ COO) ₂), [P2O5] = 2M, [TiO2] = 1,95M, Acid propionic 99%	3,22 ml d'acid propionic, 0,177ml de Ti, 20 ml de Dioxane, 1,61 ml de P2O5 (d.b.d)	6h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P2O5 - 5 TiO2	64,04CaO - 33,17P2O5 - 2,78 TiO2
G5 Ca Pro 24h	0,6589g de Calcium Propionate (Ca(C ₂ H ₅ COO) ₂), [P2O5] = 2M, [TiO2] = 1,95M, Acid propionic 99%	3,22 ml d'acid propionic, 0,177ml de Ti, 20 ml de Dioxane, 1,61 ml de P2O5 (d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P2O5 - 5 TiO2	63,93CaO - 33,28P2O5 - 2,79 TiO2
G5 Ca Pro (0,6589*2g)	1,31g de Calcium Propionate (Ca(C ₂ H ₅ COO) ₂), [P2O5] = 2M, [TiO2] = 1,95M, Acid propionic 99%	3,22 ml d'acid propionic, 0,177ml de Ti, 20 ml de Dioxane, 1,61 ml de P2O5 (d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P2O5 - 5 TiO2	63,7 CaO - 32,72 P2O5 - 3,51 TiO2
G5 Ca Pro (0,6589/2g)	0,33g de Calcium Propionate (Ca(C ₂ H ₅ COO) ₂), [P2O5] = 2M, [TiO2] = 1,95M, Acid propionic 99%	3,22 ml d'acid propionic, 0,177ml de Ti, 20 ml de Dioxane, 1,61 ml de P2O5 (d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P2O5 - 5 TiO2	0
G5 Ca Pro 30°C	0,6589g de Calcium Propionate (Ca(C ₂ H ₅ COO) ₂), [P2O5] = 2M, [TiO2] = 1,95M, Acid propionic 99%	3,22 ml d'acid propionic, 0,177ml de Ti, 20 ml de Dioxane, 1,61 ml de P2O5 (d.b.d)	24h	30°C	20%G5 - 80%Dioxane	47,5CaO - 47,5P2O5 - 5 TiO2	63,59 CaO - 33,29 P2O5 - 3,12 TiO2

Table 9 - Recap chart 1

	Concentrations	Volumes	Aging time	Temperature	Proportion precursors/solvent	%molar theoretical	%mol obtained
G5 Ca Pro 5/95	0,6589g de Calcium Propionate (Ca(C ₂ H ₅ COO) ₂), [P ₂ O ₅] = 2M, [TiO ₂] = 1,95M, Acid propionic 99%	3,22 ml d'acid propionic, 0,177ml de Ti, 20 ml de Dioxane, 1,61 ml de P ₂ O ₅ (d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	5%G5 - 95%Dioxano	47,5CaO - 47,5P ₂ O ₅ - 5 TiO ₂	44,6 CaO - 29,3 P ₂ O ₅ - 26,1 TiO ₂
G5 Ac.Fit 07/10/13 (1,04ml)	[CaO] = 0,92M, [TiO ₂] = 1,95M, Phytic acid (50%H ₂ O)	3,69 ml de CaO, 0,2 ml de Ti, 20 ml de Dioxano, 1,04 ml de Phytic acid (d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P ₂ O ₅ - 5 TiO ₂	46,01CaO - 46,57P ₂ O ₅ - 7,42 TiO ₂
G5 Ac.Fit (0,1ml)	[CaO] = 0,92M, [TiO ₂] = 1,95M, Phytic acid (50%H ₂ O)	3,69 ml de CaO, 0,2 ml de Ti, 20 ml de Dioxano, 0,1 ml de Phytic acid (b d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P ₂ O ₅ - 5 TiO ₂	68,61 CaO - 30,92 P ₂ O ₅ - 0,47 TiO ₂
G5 Ac.Fit (0,01ml)	[CaO] = 0,92M, [TiO ₂] = 1,95M, Phytic acid (50%H ₂ O)	3,69 ml de CaO, 0,2 ml de Ti, 20 ml de Dioxano, 0,01 ml de Phytic acid (b d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	20%G5 - 80%Dioxane	47,5CaO - 47,5P ₂ O ₅ - 5 TiO ₂	77,65 CaO - 12,7 P ₂ O ₅ - 9,65 TiO ₂
G5 Ac.Fit (1,04ml) 5/95	[CaO] = 0,92M, [TiO ₂] = 1,95M, Phytic acid (50%H ₂ O)	3,69 ml de CaO, 0,2 ml de Ti, 95 ml de Dioxano, 1,04 ml de Phytic acid (d.b.d)	24h	Ambiant 20°C + 13°C Dioxane/Phosphore	5%G5 - 95%Dioxano	47,5CaO - 47,5P ₂ O ₅ - 5 TiO ₂	50,9 CaO - 42,8 P ₂ O ₅ - 6,3 TiO ₂
G5 Ac.Fit (1,04ml) 2,5/97,5	[CaO] = 0,92M, [TiO ₂] = 1,95M, Phytic acid (50%H ₂ O)	1,845 ml de CaO, 0,1 ml de Ti, 97,5 ml de Dioxano, 0,52 ml de Phytic acid (d.b.d)	24h	Ambiant 20°C + 13°C Phosphore	2,5%G5 - 97,5%Dioxano	47,5CaO - 47,5P ₂ O ₅ - 5 TiO ₂	61,42 CaO - 27,23 P ₂ O ₅ - 11,35 TiO ₂

6.2. Morphology of the particles (Shape and Size)

As we already said earlier on this project, the size and shape of the particles have an important effect on the way they interact with the desired matrix during the elaboration process; in our case we assume it is PLA and we want to it those fibers to be produced by electrospinning. After consulting several studies [41], it has been demonstrated that most favorable shape would be a spherical shape. Indeed it presents the minimum contact surface between the phases, meaning a decrease of the surface tension. In this way, we can assume a better wettability between the nanoparticles and the polymeric matrix.

Through SEM and FE-SEM sessions, we are able to observe our nanoparticles. According to the images (Image 3), nanoparticles obtained using the calcium propionate

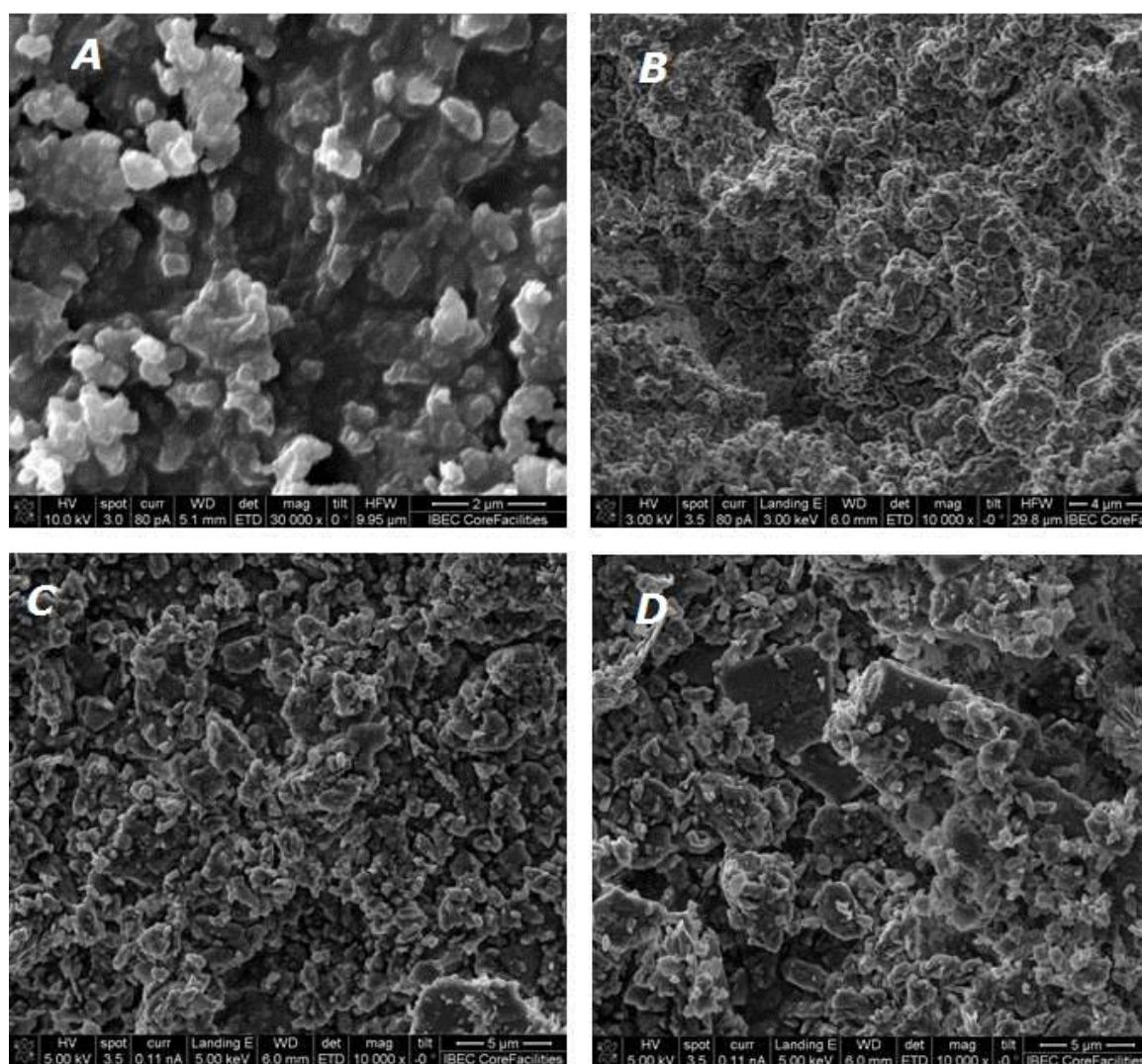


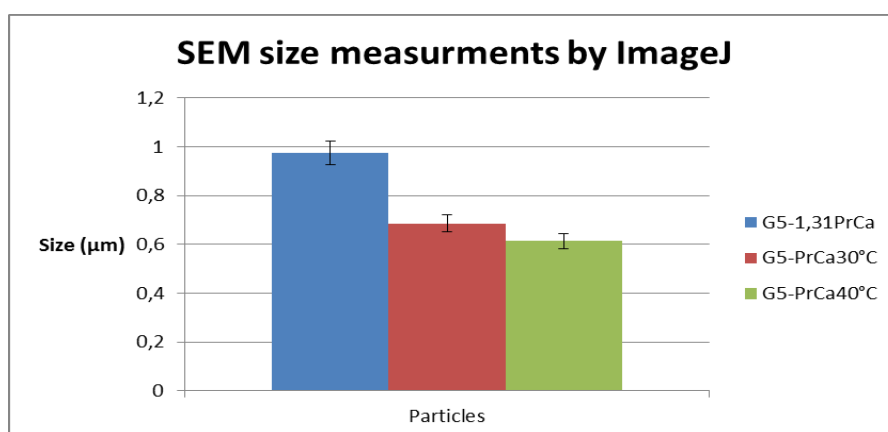
Image 3 - Calcium propionate nanoparticles imaging by SEM: a) G-5 calcium propionate 24h aging. b) G-5 calcium propionate 1,31g. c) G-5 calcium propionate 30°C. d) G-5 calcium propionate 40°C.

powder precursor show the difficulty in the control of getting perfect spherical shape. Using higher magnification (Image 3-a), we can distinguish partially round particles in the first plan, but with no real homogeneous tendency due to a general important agglomeration. Image 3-b confirms the very high amount of particles agglomerates when increasing the initial amount of calcium propionate (1,31g of initial powder instead of 0,656g). However in Image 3-c,d, the images suggest plate shape particles rather than spherical. This can be explained by a strong dependence on the growth speed, which leads to an anisotropic behavior (especially for crystalline particles).

SEM showed in previous studies [42] that bioactive glass particles in the nanoscale range had a tendency towards agglomeration.

Most studies about bioactive glass nanoparticles concerns ternary systems with silicon dioxide SiO_2 , which present a focus on the synthesis of irregularly spherical particles presented as aggregate. One paper suggests controlling the morphology of these particles using lactic acid in the sol-gel process [43].

Besides the shape, particles sizes influence the interaction with the PLA matrix as well. It has been already demonstrated that the decrease in particles size from the micro scale to the nano scale promote the embedding between the matrix and the particles. The sizes of our particles were measured by both SEM images (using the ImageJ software) and Zetasizer measurements for the most interesting and satisfactory ones. Graphic 1 shows the results obtained for the calcium propionate:



Graphic 1 - Size measurements by ImageJ – calcium propionate

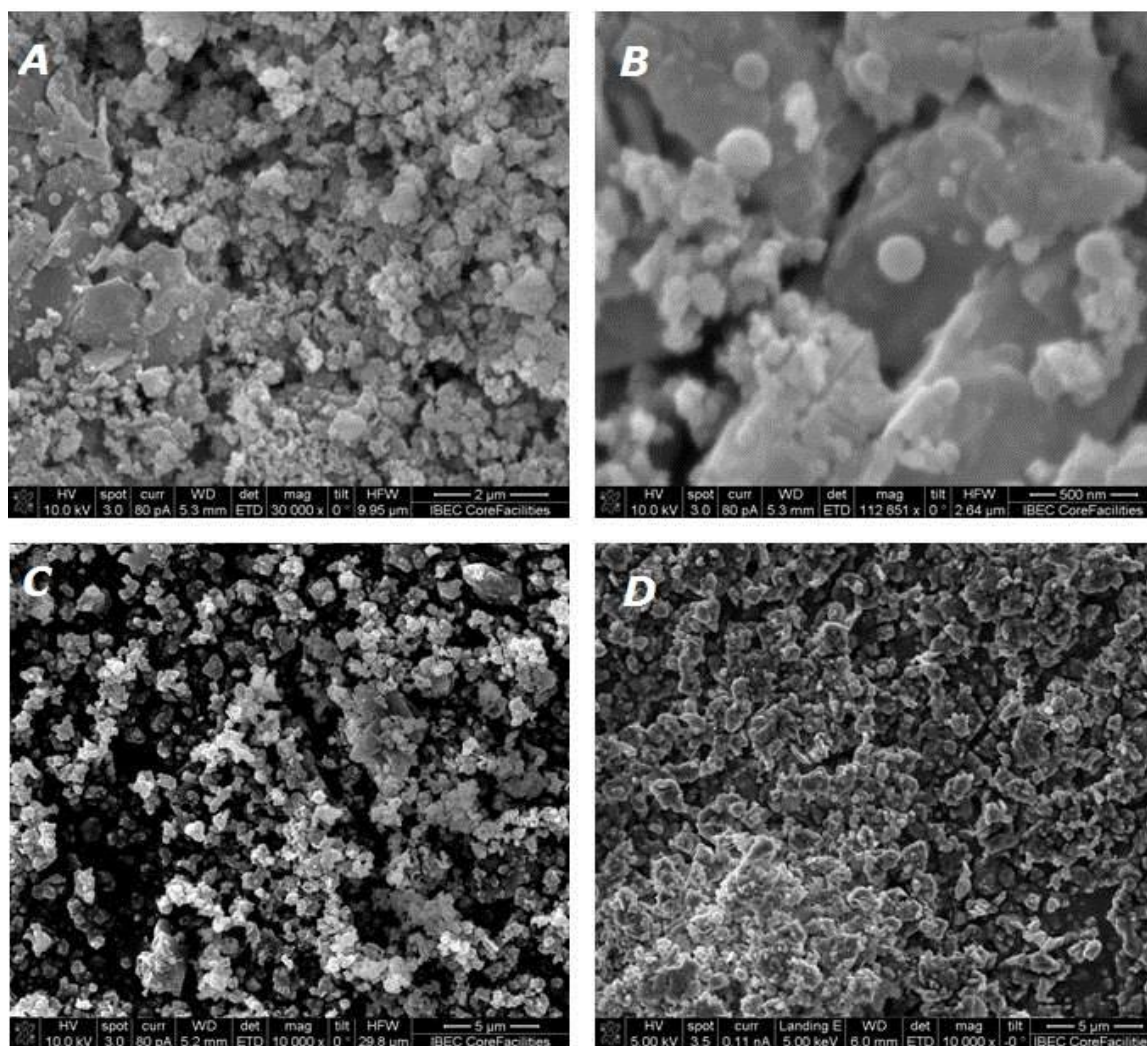


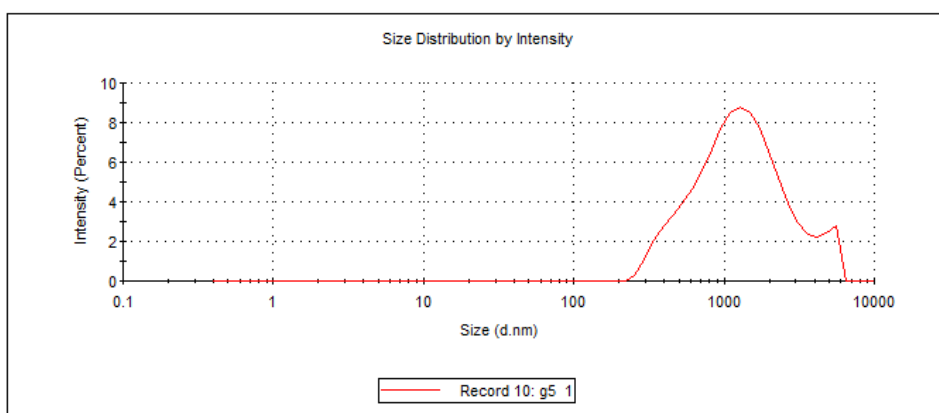
Image 4 - Phytic acid nanoparticles imaging by SEM: a) G-5 Phytic acid 20%-80%. b) G-5 Phytic acid 20%-80% higher magnification. c) G-5 Phytic acid 5%-95%. d) G-5 Phytic acid 0,1ml.

When using the phytic acid precursor, it appears on the SEM imaging (Image 4); we still have a recurrent issue concerning the agglomeration. However, very fine and perfectly spherical nanoparticles were found in the sample of the G-5 Phytic acid with ratio precursors/solvent 20-80%, see image 4-b. Contrary to what has been observed with the calcium propionate, the shape of the particles obtained with the phytic acid show better ellipsoidal-spherical behavior. According to some studies on bioactive glasses, smaller nanoparticles can be obtained by controlling carefully the rate of polycondensations, meaning slowing it down, through several parameters [44] (accurate control of temperature during the synthesis, pH, decreasing aging time, molar ratio of solvent/precursors). Unfortunately, the results don't seem to reach a common stance as the control of all the

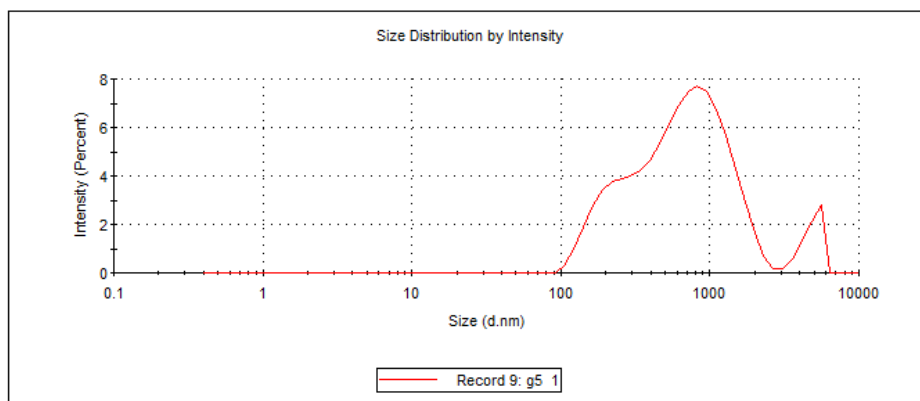
parameters engaged in the process is very challenging. After all, we clearly obtained nano scale particles using phytic acid with quite interesting shape and good dispersion size. Agglomeration still remains an issue though.

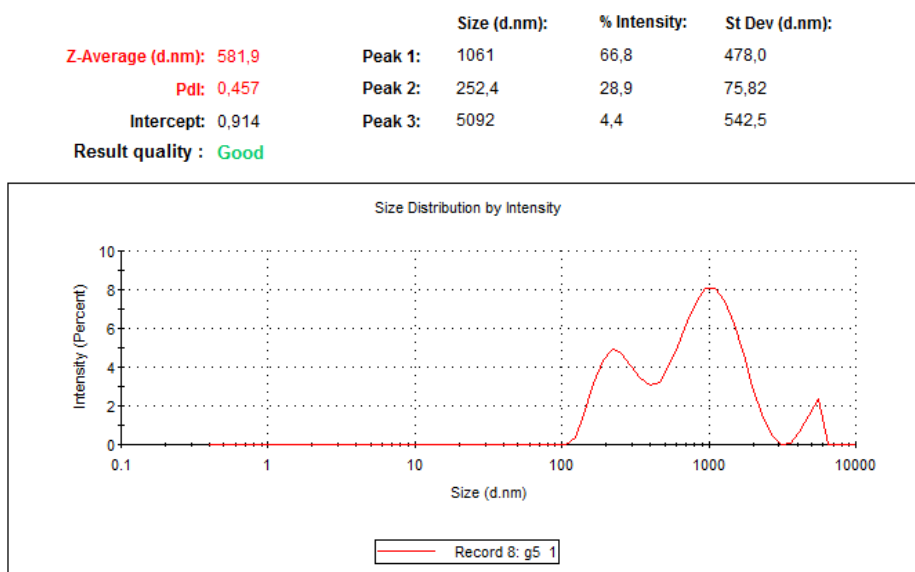
Phytic acid use show better results for the size of the particles. Indeed, as mentioned before, we performed Zetasizer and SEM imaging measurements, as followed in Graphic 2:

	Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 1123	Peak 1: 1416	92,7	876,6
Pdl: 0,374	Peak 2: 4888	7,3	579,8
Intercept: 0,901	Peak 3: 0,000	0,0	0,000
Result quality : Good			

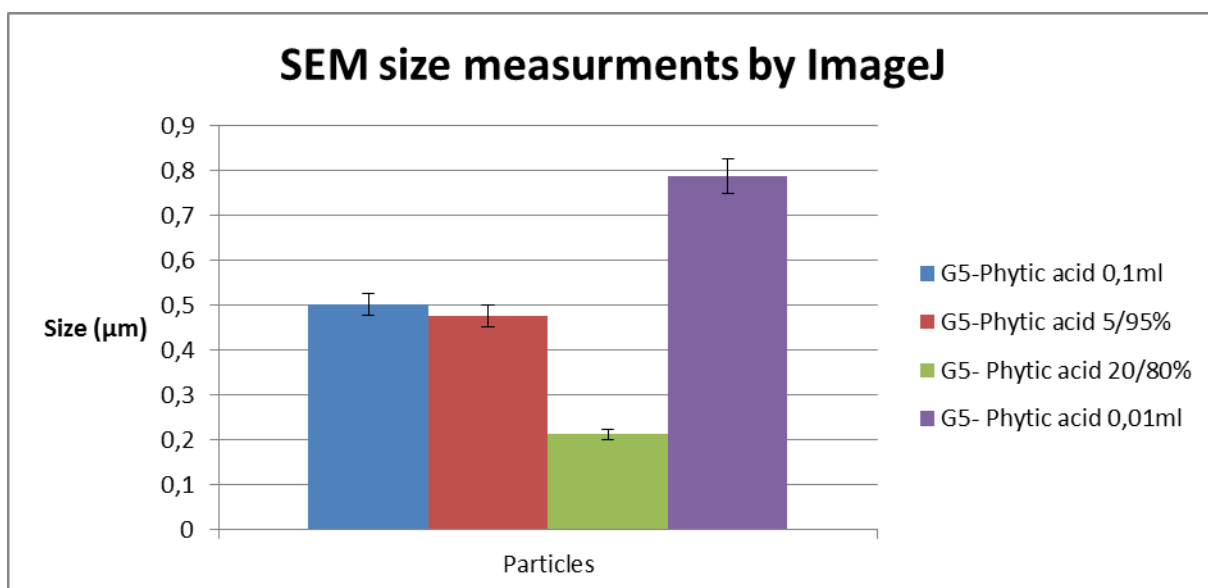


	Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 557,3	Peak 1: 736,1	93,0	486,0
Pdl: 0,434	Peak 2: 4831	7,0	716,3
Intercept: 0,912	Peak 3: 0,000	0,0	0,000
Result quality : Good			





Graphic 2 - Particles size measure Zetasizer - a) G5-Phytic acid 20-80%.b) G5-Phytic acid 5-95%. c) G5-Phytic acid 2,5-97,5%



Graphic 3 - Size measurements by ImageJ phytic acid

The first comment that we are able to do, is that, although we prepared our samples for the Zetasizer measurements in a very careful way (ethanol, absolute ethanol with ultrasounds dispersion); the results obtained (see Graphic 2-a-b-c) are too imprecise. This is due to the fact that the Zetasizer gives us a very broad range of analyses, obviously

measuring agglomerates rather than the particles themselves, especially for the Figure 4-a. Nevertheless, it gives us a good estimation of the dispersion and average size of the measured agglomerates/particles. As we can see in Graphic 2-b, we have an average size of 557,3 nm for the G5 phytic acid 5-95%, which corresponds with the results obtained through the SEM imaging measures. The measurement by the software ImageJ (Graphic 3) remains the most accurate one, we were able to measure particles with a size of approximately 71 nm.

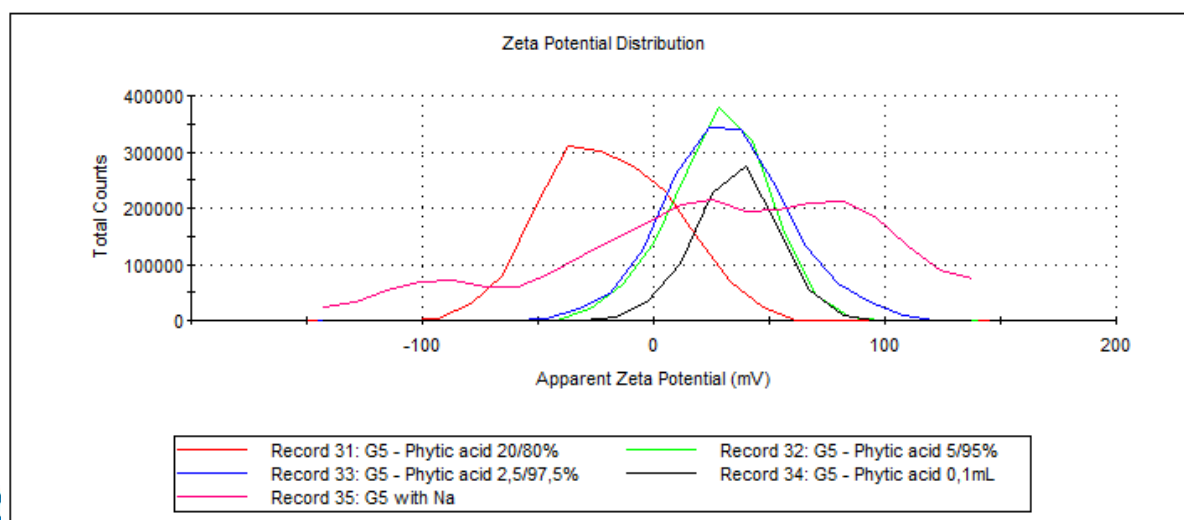
6.3. Zeta potential analysis

We performed zeta potential measurements because it is directly linked to the morphology (shape/size) of the nanoparticles for the wettability by the polymeric matrix. According to the literature [45], the main important mechanisms for a colloidal particle to have a surface charge are:

- Differential loss of ions
- Adsorption of charges species (ions and ionic surfactants)
- Ionization of surface groups

As mentioned before, the value of the zeta potential will affect the way our particles establish interactions with the matrix. Ideally, the two materials involved in the interaction have opposite surface charged, and in the better cases, two high opposite values of zeta potential.

Values of Zeta potential from our phytic acid derived particles samples with different concentrations and ratio solvent/precursors are shown in Graph 4:



Type	Sample Name	T	ZP	St Dev
		°C	mV	mV
Zeta	G5 - Phytic acid 20/80%	25	-18,9	0
Zeta	G5 - Phytic acid 5/95%	25	27,6	0,6
Zeta	G5 - Phytic acid 2,5/97,5%	25	28,75	2,95
Zeta	G5 - Phytic acid 0,1mL	25	34,1	1,4
Zeta	G5 with Na	25	43,3	0

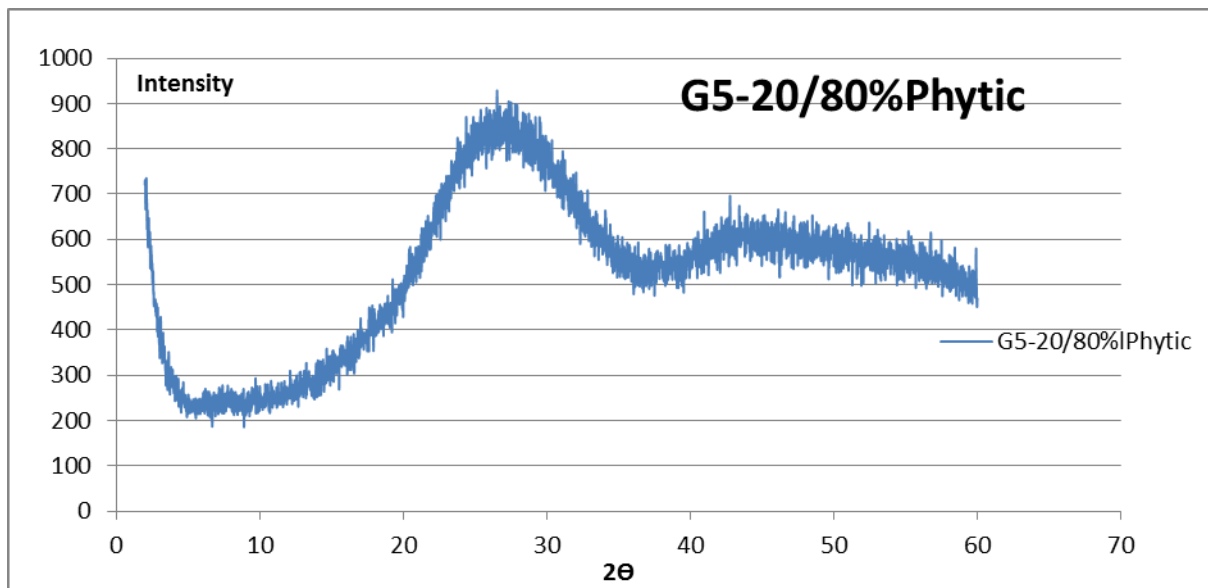
Table 11 - Zeta potential values for different samples

As it has been shown on the graphic 3 and the table 11, most of the results for potential zeta seems to have a positive charge on the particles surface, which is in accordance with our expectations. One of our results, for the G5-Phytic acid 20/80% appears to be a little bit surprising as it shows a -18,9 mV negative surface charged, which we can relate to an unexpected possible excess of phosphorus content at the surface of the particles. . Besides, we obtained correct values in terms of colloidal system stability as the rest of our data samples expressed in table 8, are included in a 28.2mV to 43.3mV range. Positive values for our samples can be explained by the presence of both Ca^{2+} and Ti^{4+} ions at the surface of the nanoparticles in comparison with the negatively charged phosphate ions [33].

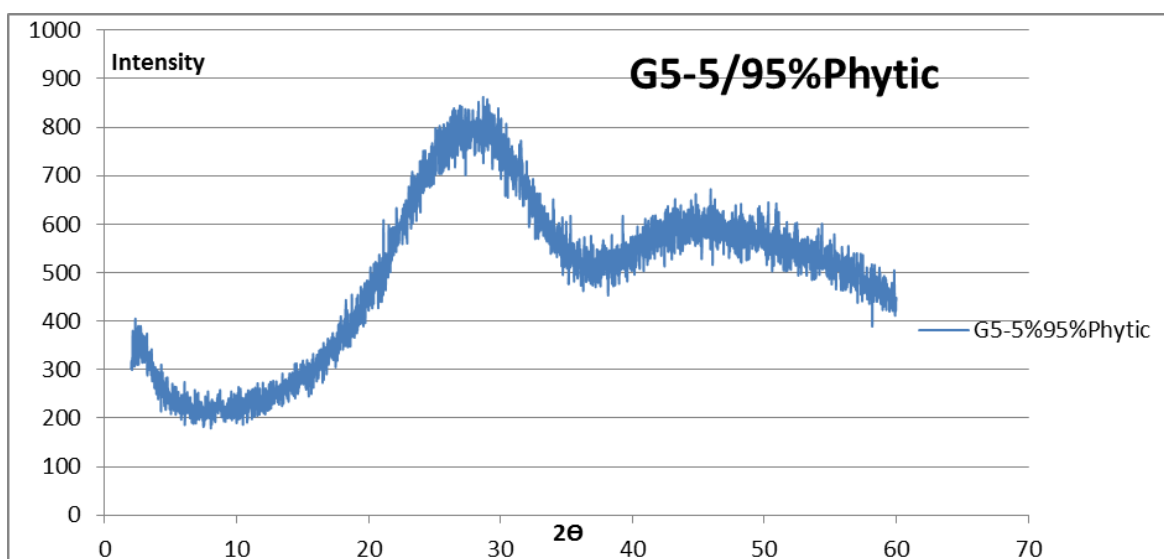
A slight tendency has been observed during these measurements towards the value of the positive charges according to the differences between the samples. Indeed we were able to observe that increasing values have been detected when the ratio solvent/precursors is actually decreasing.

6.4. Crystallinity of the particles

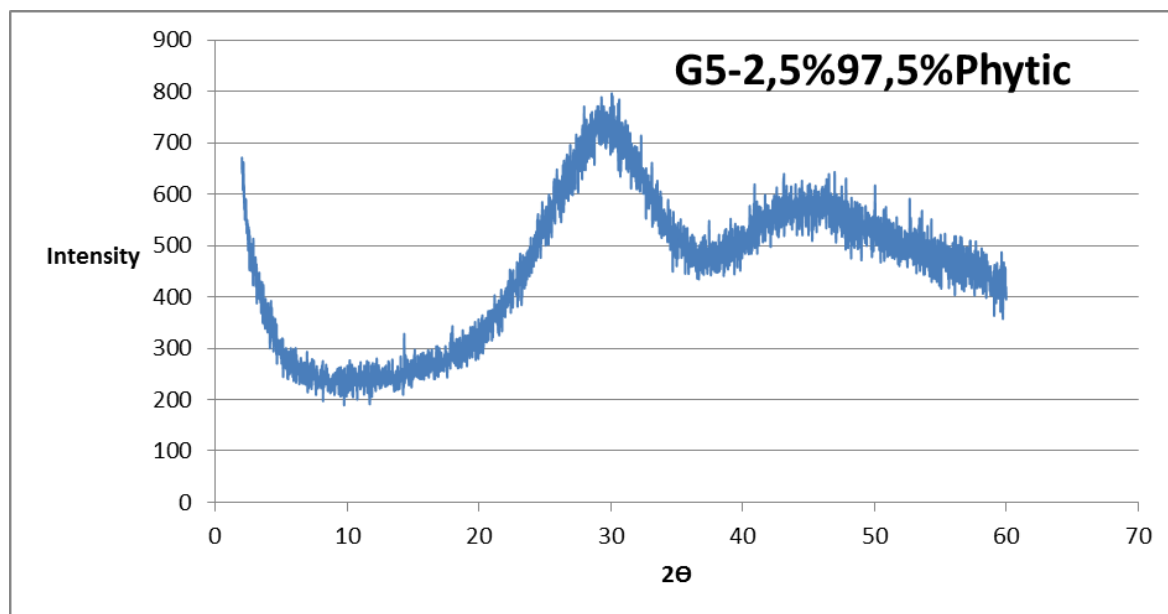
We carried out X-ray diffraction on a range of five different samples. This characterization method has been performed preferably as a control experiment rather than to determine crystalline structure of the material. Indeed, as we produced glasses via the sol-gel method, these assays are to confirm the amorphous structure of our nanoparticles. This information about the structure will approve the degradable nature of our glasses.



Graphic 5 - X-ray diffraction spectrum G5 - Phytic acid 20/80%



Graphic 6 - X-ray diffraction spectrum G5 - Phytic acid 5/95%



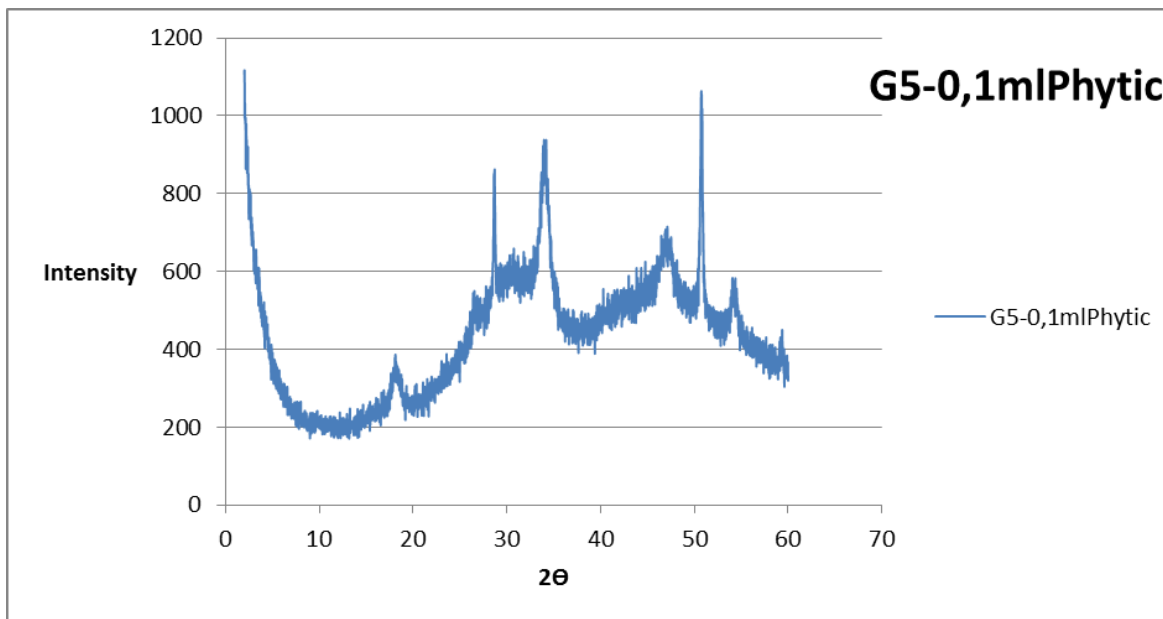
Graphic 7 - X-ray diffraction spectrum G5 - Phytic acid 2,5/97,5%

When X-ray radiation impacts the studied material, in our case nanoparticles powder, the lattice spacing of the molecules in the solid tends to behave as a diffraction grating. Because the distance travelled by the beam depends on the plane of reflection, the diffraction beam displays different phases. Two types of interferences are encountered; constructive (increase of the intensity – diffracted waves are in phase) and destructive (no intensity – diffracted waves out of phase [46]).

The X-ray spectrum analysis can be used to confirm the amorphous nature of our powder samples. Unlike crystalline materials, amorphous materials do not have long range order and in this way the spectrum does not present the common peaks correlated to crystalline phases. According to our diffraction patterns, graphic 5, 6 and 7 show respectively three broad “halo” with one or only few maxima. The literature associated [9] gives us the required knowledge to understand and interpret our results. These depicted spectrums confirm us that our particles G5-20/80%, G5-5/95% and G5-2,5/97,5% are amorphous materials, even though we cannot identify the material being analyzed.

Those results are in accordance with our expectations and totally logical as we did not perform any heat treatment after the synthesis. Therefore we obtained a glassy structure, which will clearly suits the desired rate of degradation, rather than a crystalline structure, which would take more time to degrade.

In the case of G5-Phytic acid 0,1 mL (Graphic 8), we can observe several peaks at $2\theta=28,33$ and 51° , suggesting that a calcium phytate crystalline phase was encountered in the powder. The very low amount of titanium can be the reason, as we have a final composition of almost binary calcium phosphate glass.

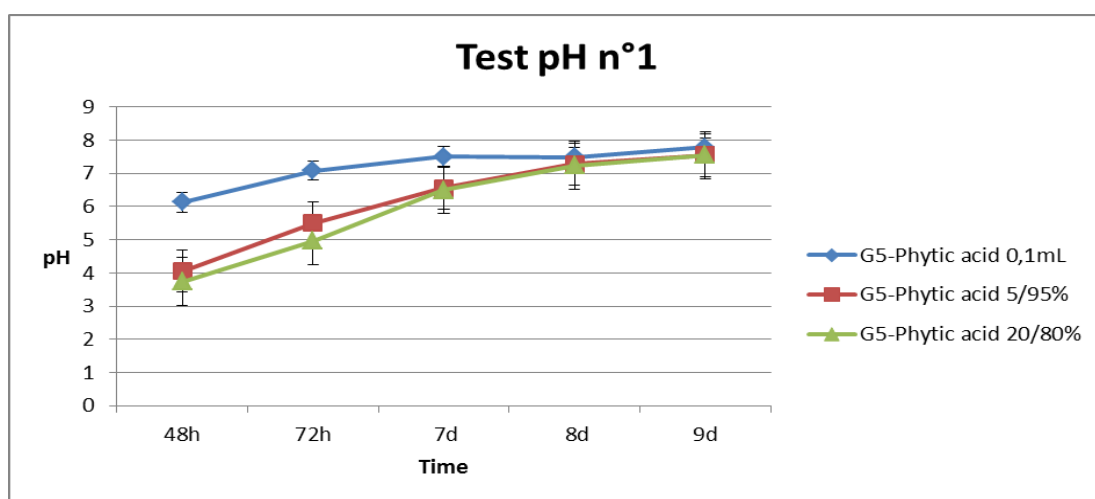


Graphic 8 - X-ray diffraction spectrum G5 - Phytic acid 0,1ml

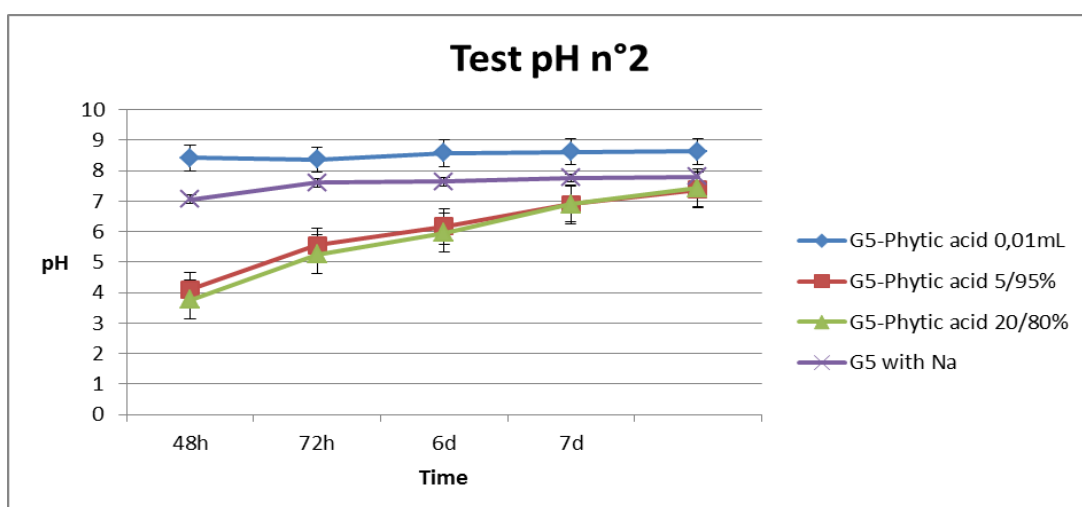
The use of the phytic acid as the phosphorus precursor shows definitely a good advantage: previous studies [47] have shown that a controlled hydrolysis of the glass sol precursor is relevant towards the obtaining of typical amorphous structure for embedding calcium-phosphate nanoparticles. This makes the phytic acid a suitable phosphorus precursor for our particles synthesis as it has a self-catalysis behavior towards hydrolysis and condensation in the sol-gel process [32].

6.5. Degradability

The main purpose of these nanoparticles is the release of ions. When immersed in an aqueous medium, the release of calcium, phosphorus, and titanium ions occur, it suggests a change in the pH value of the medium, which can affect the parameters that we try to control. This release is studied as it is important to have knowledge on how fast and in which amount these ions pass from the glass through the body, especially for our major goal, which is precisely the release of calcium (as we mentioned before, important for angiogenic properties [48]).



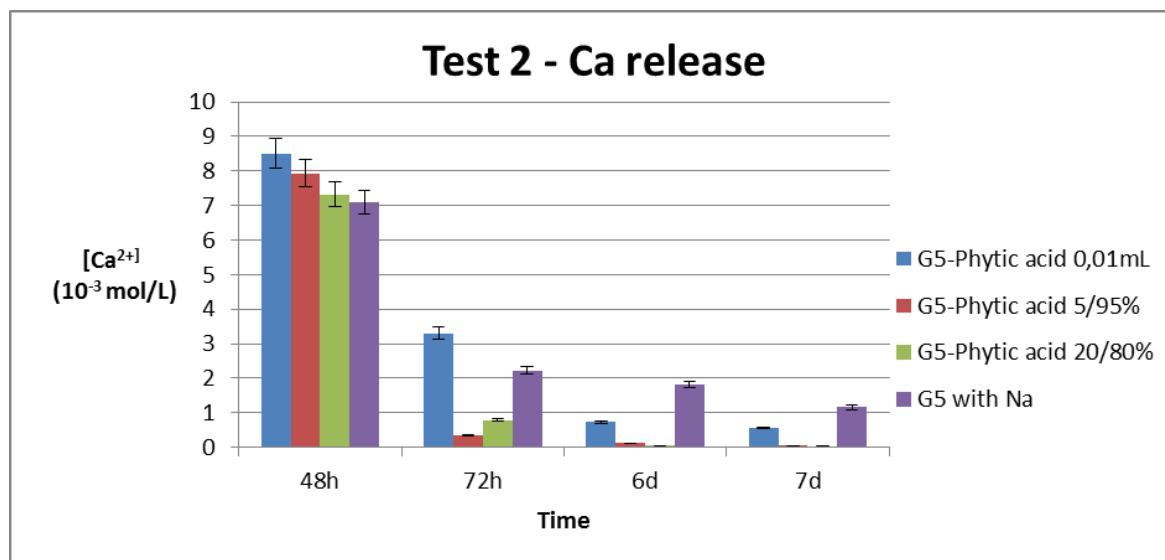
Graphic 9 - pH measurements (48h to 9 days) – Test n°1



Graphic 10 - pH measurements (48h to 7 days) – Test n°2

In order for living cells to survive, a specific pH range has to be defined. This pH value is known as the physiological pH and is close to 7,4. Consequently, it means that values far from this 7.4 pH may induce rejection from the cells. The graphic 9 and 10 show the results obtained for different powder samples: G5 particles using phytic acid with a ratio solvent/precursor of 5/95% and 2,5/97,5% express the most acidic behavior after 48h, with values included between 3,75 and 5,50 until 72h after immersion, which is obviously too low in comparison with the physiological pH. However these results can be directly correlated to the amount of phosphorus contained in the different samples: particles containing high amount of phosphorus (typically more than 40% of molar ratio) are the ones of which the pH values stay under 6 for the first 5 to 6 days. With a molar ratio of only 12.7% in P_2O_5 , we can observe that the values of pH for the G5-Phytic acid 0,01mL are above 8 since the beginning of the assays.

The pH differences depend directly on the composition of our powders indeed. This is due to the reactions between the ions liberated from the G5 and the HEPES medium. Liberation of $P_2O_4^{2-}$ leads to a low acidic pH (formation of H^+ ions) and simultaneously liberation of Ca^{2+} promotes an increase of the pH (due to formation of HO^- ions). This is why a good balance of both entities will lead to a suitable pH for the cells.



Graphic 11 - Ca release measurements

We performed calcium release measurements as observed in the graphic 10. The calcium has to be released at a suitable rate in order to promote the formation of new blood vessels. For timetable issues, we only carried out these assays on a 7 days basis.

The graphic 11 clearly expresses a high release of Ca^{2+} during the 3 first days for the powder samples with important calcium molar ratio (G5-Phytic acid 5/95% and G5-Phytic acid 0,01mL especially). We decided for the second test to perform this assay on a “regular” G5 with sodium content in order to get a comparison with our produced particles. It appeared that the release for the G5 with sodium is more homogenous in time. After having calculated the cumulative calcium release data, we were able to obtain the release rate for each material:

	Rate of release (mmol.L-1/day)
G5-Phytic acid 0,01mL	1,87
G5-Phytic acid 5/95%	1,21
G5-Phytic acid 20/80%	1,17
G5 with Na	1,76

Table 12 - Rate of release of particles samples for 7 days

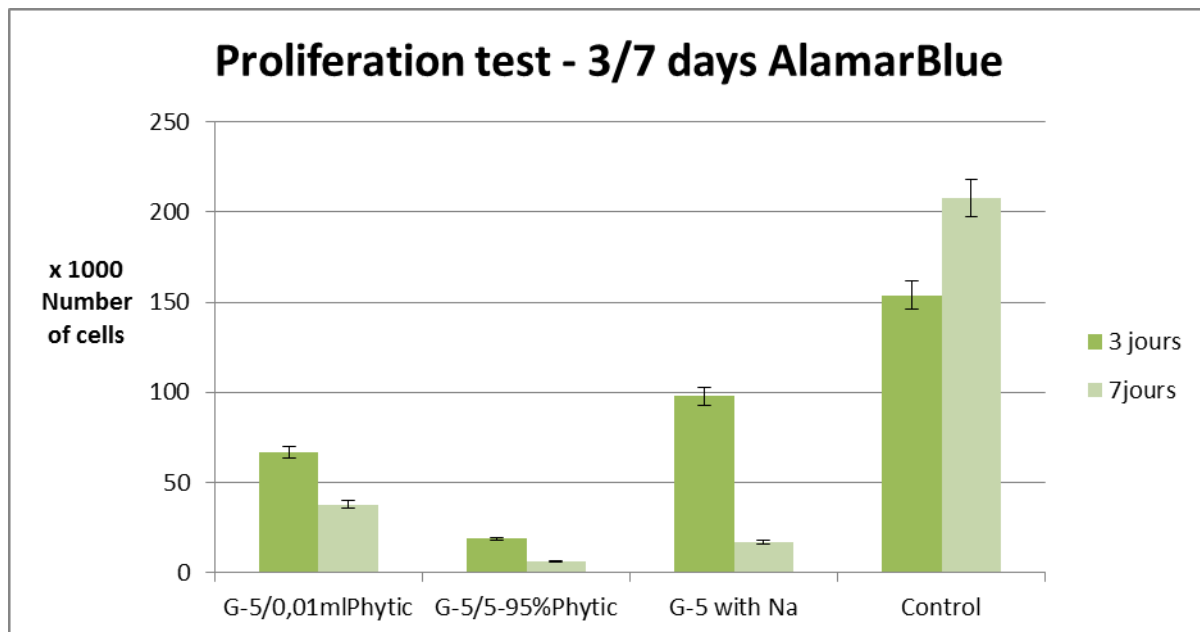
The table 12 shows that the rate of calcium released is, as expected, composition dependent. Besides, the G5-Phytic acid 0,1mL is a good example of the important role of the titanium content. According to our EDX results, G5-Phytic acid 0,01mL expressed a higher amount of TiO_2 (molar ratio 9.65%) for only 12% of P_2O_5 , which is directly linked to its apparent “control” release of Ca^{2+} . We know for a fact that the titanium is the stabilizer of the vitreous network in our case, which is why the 5% or more required amount of TiO_2 has to be precisely respected in order to get a suitable ions release. However such a low amount of P_2O_5 is due to the low quantity of precursor injected during the synthesis, cells assays will not respond as expected towards a possible reduced toxicity of our products, the main compound being the CaO with a molar ratio of 77,65%, as we know obtained with the 2-methoxyethanol.

The release of calcium when the hybrid material is obtained by electrospinning would be worth considering, as we know that calcium release can reach levels of cytotoxicity

in the case of some studies [50]. But as the results show no calcium release higher than 10 mmol/L, it does not seem to have any issues once embedded in the polymeric matrix.

6.6. Cell assays – Proliferation tests

After having calculated the calibration curve from the AlamarBlue® product, as mentioned before, we were able to analyze the data obtained for our different powder samples on rat mesenchymal cells (Graphic 12):



Graphic 12 - Proliferation test 3/7 days using AlamarBlue

As we know how difficult it was to control the pH from our powder samples, as well as the release of calcium, it is without surprise that we attest the non-effective aspect on the cells assays, at least for the G5-phytic acid 0,01mL and for the G5-phytic acid 5/95%. The low pH is precisely the reason for such low values in the case of the G5-phytic acid 5/95%, combined with the fact that the calcium precursor used with phytic acid was obtained in 2-methoxyethanol and as we know is cytotoxic. When the pH of the sample is higher (above 7) , in the case of G5-phytic acid 0,01mL for example, we can observe that it reaches more than twice the number of cells than with the G5-phytic acid 5/95%, even though the very low

amount of P_2O_5 misrepresent what we could have expected. The same interpretation can be done for the G5 with sodium. However we have to notice still an important difference compared to the control, which is obviously due to the toxicity of the byproducts of both 2-methoxyethanol and ethanol from the precursors used.

7. Conclusion

Throughout this project, the sol-gel method was used to produce glass nanoparticles with different precursors. The chemical compositions of the glasses were adjusted according to the initial standards and previous studies. It appeared that the use of different precursors in order to prevent cytotoxicity was possible, we carried out interesting syntheses to obtain our types of G5 nanoparticles with a theoretical composition of 47,5% CaO - 47,5% P₂O₅ and 5% TiO₂.

These particles were suggested to have a spherical shape and to be positively charged in surface for a further good impregnation with PLA matrix for example. SEM imaging allowed establishing the G5 nanoparticles size range from 200 nm to 700 nm depending on the different samples, and the use of the Zetasizer attested a positive surface charge for the particles obtained with the phytic acid precursor. Besides, the effects of G5 nanoparticles in medium for cell proliferation assays were measured. pH and AlamarBlue® tests indicated that the phosphate ions release from the breaking network induced an significant acidification effect on the medium. A better control of the synthesis using the calcium propionate precursor has to be considered, as very low quantities of particles were produced, too insufficient for performing most of the regular characterization methods.

Unfortunately the cell proliferation assays did not meet our desired expectations, which is mostly due to very broad results in terms of composition. Once the synthesis parameters will be more precisely controlled, better morphology and compositions of particles will allow us to fully understand and interpret the non-toxic feature of byproducts released by these new precursors.

Thus, further and deeper studies regarding these precursors would be of significant interest. Despite a lack of publications concerning this step of organic byproducts release in the body, it would be in my opinion interesting to do some research towards the concurrent use of both calcium propionate and phytic acid precursors in the same synthesis (obviously paying attention the pH) and towards the possibilities to use only one precursor for both calcium and phosphorus entities as well. For instance, phytic acid calcium salt (currently available at Sigma-Aldrich) would be very interesting in this way, but sadly too expensive.

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Spending

Economic evaluation of the project

Perform an economic evaluation of a research project is a tricky task, since it can only be a global evaluation. However, we try to quantify these costs according to three main headings; material costs, personnel and equipment costs.

Material costs

The costs due to the material needed for the project are presented below.

Costs associated with cleaning glasses, laboratory samples, tools, and solvents used:

Solvents	Provider	Quantity	Price (€/L)	Final cost (€)
Ethanol	Panreac	2L	16.21	32.42
Acetone	Panreac	2L	22.55	45
Absolute ethanol	Panreac	0.5L	16.87	8.4

Products	Provider	Units	Price per unit	Final cost (€)
Pipette	DB	30	0.35	10.5
Syringes	DB	100	0.1	10
Gloves	Naturflex	400	0.04	16

Costs associated with the sol-gel synthesis:

	Provider	Quantity	Price (€/L) or (€/kg)	Final cost (€)
1,4-Dioxane	Sigma Aldrich	0.6L	116	69.6
2-Methoxyethanol	Sigma Aldrich	250mL	122	30.5
Metallic calcium	Sigma Aldrich	10g	287	2.87
Phosphorus pentoxide	Sigma Aldrich	10g	84	0.84
Titanium	Sigma Aldrich	10mL	135	1.35

isoproxide					
Calcium propionate	Sigma Aldrich	10g	54.6		0.55
Propionic acid	Sigma Aldrich	30mL	32.5		0.97
D-Pantothenic acid hemicalcium salt	Sigma Aldrich	20g	74		1.48
Phytic acid 50% (w/w) H₂O	Sigma Aldrich	10mL	193		1.93
Phytic acid sodium salt hydrate	Sigma Aldrich	1g	1452		1.452

Prices of these chemicals and solvents were obtained by catalogs and online webstores of the supply companies such as Panreac and Sigma Aldrich.

Costs associated with staff

These costs are evaluated very approximately. The project has involved the intervention of several people; we tried to make a total cost for all interventions:

Personnel	Price per hour (€)	Total Price (€)
Project manager	50	100
Project supervisor	30	900
Specialized technicians	20	625
Junior engineer	9	7200

Costs of equipment

It is an approximate cost from the use of equipment and services during the whole time of research:

Equipment	Price per unit (€)	Total Price (€)
SEM - FESEM	37-45/h	296-360
DRX	10/unit	50
Zetasizer	-	-
pH & ion meter	5/h	100
Cell culture	45/h	675
Sample coatings	7/unit	60

The SEM, FE-SEM and DRX do not belong to the IBEC laboratory, so the estimated prices are variable, because such analyses requires external equipment.

Total cost of the project

The final cost of the project is calculated by adding each cost. However, costs due to laboratory expenses such as electricity, water, basic equipment, office supplies and more have to be estimated as well. According to previous costs estimated for the same type of project, these costs are estimated as 10% of the sum of staff, equipment and products.

The following table shows the final cost:

Type	Total price (€)
Price of the material	234
Price of staff interventions	8825
Equipment	1213
10% total expenses	307
Total cost of the project	9366

The final cost the project, adding each section, is calculated to be: 9366€.

The estimation of this evaluation is thus 10,000€.

Environmental evaluation

Despite the fact that this 6-month project will not revolutionize the bioengineering field, it has however a contribution to the environmental impacts. Indeed, such a work has the possibility to bring interest and to lead to further researches for biomedical applications, especially in the area of nanofibers scaffolds for bone regeneration.

Apart from this point, we have to consider the impact generated by the use of laboratory equipment, during the whole process of material synthesis, but during the characterization as well. Electrical consumption has to be taken into account, but some efforts have been made concerning schedule management to prevent the use of any device during long period (stirring syntheses during the night for example).

Furthermore, an important part of this project was the synthesis of materials, using especially chemicals compounds. In order to preserve the environment, we intended as much as possible to follow the laboratory guideline. For example 3 different trashes are used to sort waste selectively: Liquid waste (acids, bases, halogens or non-halogens), bio-waste (material used for cell culture) and finally a more general contaminated waste (syringes, gloves, tubes, etc...).

Finally, we know that a few products can be reused. Therefore we proceeded to the “recycling” of products such as ethanol or dioxane, for which distillation is possible. Dioxane being the solvent used for all syntheses, significant savings was made.

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